METHOTREXATE AND RISK OF CUTANEOUS MELANOMA

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This is only a tiny piece in a giant puzzle.

ABSTRACT

Methotrexate (MTX) is an anti-inflammatory and immunosuppressive drug commonly used to treat psoriasis, psoriatic arthritis and rheumatoid arthritis. Cutaneous malignant melanoma (CMM) is a common and dangerous type of skin cancer and in recent decades a noteworthy increase in incidence has been observed. In Sweden, CMM is the fifth most common form of cancer in both men and women. This type of cancer is more frequent among patients with an impaired immune system such as organ transplant recipients (OTRs) who are treated with immunosuppressive drugs to prevent rejection of the transplanted organ. The use of MTX, has previously been associated with an increased risk of CMM in an Australian investigation.

The purpose of this thesis was to study the association between MTX and the risk of CMM.

In Paper I, a retrospective comparative cohort study was conducted, comprising all Swedish individuals over 18 years with at least one filled MTX prescription in the time period 2005-2014 (MTX-exposed). For each MTX-exposed patient, five age- and sex-matched MTX-unexposed individuals were selected (MTX-unexposed). The risk of CMM was elevated among MTX-exposed subjects, but this risk increase was lower than previously observed and hardly relevant in clinical practice.

To further investigate a possible association between MTX and CMM, a dose-response analysis was performed. Paper II used the cohort above and analyzed whether increased MTX doses elevated the risk. In summary, no conclusive dose-response relationship between MTX and CMM was observed.

Paper III investigated whether CMM that occurred in MTX-exposed patients caused an increased mortality compared to CMM occurring among the MTX-unexposed individuals. MTX-exposed patients had an increased risk of melanoma mortality. This observation was robust, after adjusting for melanoma stage at diagnosis.

Paper IV investigated patients who had already had CMM and exposed to MTX after the first CMM diagnosis. The risk of a new CMM among these patients was not increased compared to a corresponding MTX-unexposed group.

Paper V was performed using individuals from a cohort of psoriasis patients. Previously cancer-free psoriasis patients who developed CMM and psoriasis patients who had not developed CMM at the corresponding date were compared. The proportion

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exposed to MTX in each group did not differ significantly.

In *Paper VI*, the dermoscopic appearance of CMM that occurred in OTRs was investigated. The melanoma-specific features in this group were compared to age- and sex-matched controls. When analyzing the results, no differences could be observed. Nevertheless, these results are limited due to a small sample size and should instead be regarded as an invitation to more investigations.

In conclusion, this thesis has shown that CMM is unlikely to be associated with the use of MTX and the dermoscopic appearance of CMM in immunosuppressed patients does not seem to differ from those of immunocompetent individuals.

SAMMANFATTNING PÅ SVENSKA

Metotrexat (MTX) är ett anti-inflammatoriskt och immundämpande läkemedel som ofta används för behandling av bland annat psoriasis samt ledgångsreumatism. Melanom är en vanlig och farlig form av hudcancer och de senaste decennierna har en kraftig ökning observerats i flera västerländska befolkningar. I Sverige är melanom den femte vanligaste cancerformen bland kvinnor och män. Melanom är vanligare bland patienter med nedsatt immunförsvar såsom organtransplanterade som kroniskt står på immundämpande läkemedel för att förhindra avstötning av sitt transplanerade organ. Användning av MTX har, i en tidigare australiensisk studie, kopplats samman med en ökad risk att utveckla melanom.

Det övergripande syftet med den här avhandlingen var att närmare studera en eventuell association mellan MTX och risken att utveckla melanom.

Delarbete I var en retrospektiv komparativ kohortstudie som omfattande alla svenska individer över 18 år med åtminstone ett läkemedelsuttag av MTX i tidsperioden 2005-2014 (MTX-exponerade). För varje MTX-exponerad patient, valdes fem ålders- och könsmatchade individer ut. Dessa patienter hade inte blivit exponerade för MTX (MTX-oexponerade). Andelen patienter med melanom i respektive grupp beräknades med hjälp av det svenska cancerregistret. Risken för melanom var ökad bland MTX-exponerade individer. Däremot var den uppmätta risken lägre än vad som tidigare observerats och knappast relevant i kliniken.

För att ytterligare styrka ett eventuellt samband mellan MTX och risken för melanom genomfördes en dos-responsanalys. *Delarbete II* använde kohorten ovan och analyserade om stegrade doser MTX ökade risken för melanom. Sammanfattningsvis fanns det inget tydligt dos-responssammanhang mellan MTX och risken för melanom.

Delarbete III studerade om melanom som uppstod hos MTX-exponerade individer var associerad med en ökad melanom-orsakad dödlighet jämfört med de melanom som uppstod hos MTX-oexponerade individer. Melanomstadium vid diagnostidpunkt skiljde sig inte mellan grupperna, däremot hade MTX-exponerade individer en ökad risk för melanom-dödlighet.

Delarbete IV studerade patienter som redan haft melanom och som blivit exponerade för MTX efter första melanomdiagnos. Risken för ett nytt melanom bland dessa patienter var inte stegrad jämfört med en motsvarande MTX-oexponerad grupp.

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Delarbete V utfördes bland en kohort bestående av psoriasispatienter. Tidigare cancerfria psoriasispatienter som utvecklat melanom samt psoriasispatienter som inte utvecklat melanom vid motsvarande datum jämfördes. Det var ingen skillnad i andelen MTX-exponerade i respektive grupp.

I delarbete VI studerades dermatoskopiska karaktäristika på melanom som uppstått hos organtransplanterade patienter. Utseendet hos dessa jämfördes med ålders- och könsmatchade kontroller. Några säkra skillnader kunde inte observeras.

Sammanfattningsvis har denna avhandling visat att melanom sannolikt inte är kopplat till användning av MTX. Resultaten är av värde för läkare som överväger att sätta in eller följer patienter med MTX-behandling. Vidare verkar inte melanom hos immunsupprimerade patienter skilja sig dermatoskopiskt jämfört med melanom hos andra individer.

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals. In the thesis frame they are referred to as *Papers I-VI*. The manuscripts can be found at the end of the thesis.

I. Polesie S, Gillstedt M, Sönnergren HH, Osmancevic A, Paoli J.
Methotrexate treatment and risk for cutaneous malignant melanoma: a retrospective comparative registry-based cohort study.
Br J Dermatol. 2017 Jun;176(6):1492-1499.

II. Polesie S, Gillstedt M, Paoli J, Osmancevic A.
Methotrexate Exposure and Risk of Cutaneous Malignant Melanoma: No Evidence of a Dose-response Relationship.
Acta Derm Venereol. 2018 Oct 10;98(9):888-895.

III. Polesie S, Gillstedt M, Paoli J, Osmancevic A.
Methotrexate and melanoma-specific mortality.
J Eur Acad Dermatol Venereol. 2019 Mar; 33(3):e123-e125 Oct 25.

IV. Polesie S, Gillstedt M, Paoli J, Osmancevic A.
Methotrexate treatment in patients with a history of cutaneous melanoma and the risk of a consecutive primary melanoma: A national retrospective registry-based cohort study.
J Am Acad Dermatol. 2017 Jul;77(1):161-163.

V. Polesie S, Gillstedt M, Paoli J, Osmancevic A.
Methotrexate treatment for psoriasis patients and risk of cutaneous melanoma: a nested case-control study.
In manuscript (submitted).

VI. Polesie S, Gillstedt M, Zaar O, Osmancevic A, Paoli J.
Dermoscopic Features of Melanomas in Organ Transplant Recipients.
Acta Derm Venereol. 2019 Jul 12. [Epub ahead of print].

CONTENT

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ABBREVIATIONS

ATC	Anatomical Therapeutic Chemical
CI	Confidence interval
CMM	Cutaneous malignant melanoma
DMARDs	Disease-modifying antirheumatic drugs
FA	Folic acid
GTT	Gestational trophoblastic tumor
HD-MTX	High dose methotrexate
HR	Hazard ratio
LD-MTX	Low dose methotrexate
LPD	Lymphoproliferative diseases
MTX	Methotrexate
NMSC	Non-melanoma skin cancer
OR	Odds ratio
OTR(s)	Organ transplant recipient(s)
Pso	Psoriasis
PsoA	Psoriatic arthritis
RA	Rheumatoid arthritis
SIR	Standardized incidence ratio
WHO	World Health Organization

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1 INTRODUCTION

1.1 RISK

Risk is best defined as the *"probability of an event during a specified period of time"*.[1] Therefore virtually any action in life bears a risk. Crossing a street as a pedestrian increases the risk for being involved in a car accident whereas showering, in particular with a bar of soap, will increase your risk of falling and having serious injuries. Having said that, most of us cross roads and shower every day. In everyday life, it is too time-consuming to reflect on all the risks that constantly surround us. It is safe to say that human risk management is deeply rooted in our behavior. Perhaps, when we fill prescriptions of pharmaceutical drugs, we are more cautious and vigilant, not only to whether the drug is effective, but also of potential side effects. Nevertheless, if we have a serious illness, we are usually more willing to risk potentially quite severe side effects, as long as the treatment will cure us or at least relieve our symptoms. As an example, most patients would risk significant side effects with chemotherapy in order to improve their chances of long-term cancer survival. On the other hand, a patient might be more reluctant to take unnecessary medications when the disease course is less hazardous such as a common cold that resolves even without treatment. Needless to say, weighing benefits and risks in this setting can vary significantly between individuals. As pharmaceutical drugs are among the most imperative tools in medicine, expanding the knowledge on how drugs work in the human body including the panorama of their potential side effects (i.e. pharmacovigilance) is important and might ultimately influence how physicians practice

medicine. Particularly useful drugs that have been associated with negative events warrant specific attention so that physicians can get a better awareness when weighing the benefits and harms. Importantly, investigations in this field contribute to scientific hypothesis testing and hypothesis generation, which may challenge the *status quo*.[2]

1.2 RELEVANT AUTHORITIES AND DATABASES

In Sweden, suspected side effects of pharmaceutical drugs are reported to the Swedish Medical Products Agency by healthcare professionals, consumers and patients.[3] The reports are registered in the Swedish side effect database and used for the authority's continuous pharmacovigilance, statistics and research. The side effect reports collected by the Swedish Medical Products Agency are also forwarded to the European Medicines Agency[4] that is responsible for the development, maintenance and coordination of the European side effect database[5] (EudraVigilance). The most common side effects of a given drug are usually detected by previous clinical trials and are therefore known at the time of the drug approval. Nevertheless, the knowledge of rare side effects is usually far more limited. Reporting these uncommon side effects is therefore crucial for monitoring and detection of previously unknown risks related to the drugs. Sometimes these risks only emerge after drug approval, when the drug is used in a larger and more varied patient group. Anyone who works within health and medical care should report any suspected side effect. It is particularly

important to report serious and/or previously unknown side effect(s). A side effect (also referred to as an adverse effect or adverse reaction) is usually defined as all the negative effects of drugs. By definition, a side effect is considered serious if it matches any of the following criteria: life-threatening; causes hospitalization or prolonged hospital care; leads to disability or any other medically important event; causes deformity or leads to death. If a drug is found to be linked to the development of cancer, it would be considered a serious side effect. Personally, I have filed a couple of adverse event reports during my career as a dermatologist. Even though the report can be sent electronically, it certainly is time-consuming especially when you are already late for the next patient appointment. Despite being fundamentally important to our health care system, physicians in everyday clinical practice, as you will see later in this thesis, do adequately prioritize these reports.

1.3 THE DRUG DEVELOPMENT PROCESS

The path from a newly discovered molecule, that eventually might serve as a pharmaceutical drug, is extensive. The drug development process is divided in five steps[6]: *step 1)* discovery and development; *step 2)* preclinical research; *step 3)* clinical research; *step 4)* drug review by authorities and *step 5)* post-market drug safety monitoring. *Step 3* is when a drug is first tested clinically on patients. This stage is further divided into 4 phases that thoroughly assess safety, dosage and efficacy. As one can imagine, the

vast majority of drugs fail at some stage and therefore do not make it to the market. Having said that, it is critical to remember that the patients included in the clinical trials do not necessarily reflect all the patients that ultimately will be candidates for the drugs. After a first approval, each national health care system must approve the drug for the use in that particular country. At this stage, the overall knowledge about the drug, including side effects is modest.[2] However, waiting for all evidence to emerge and postponing the approval means that you halt the process of introducing an efficient drug that could be profoundly useful for selected patient groups. Therefore, post marketing investigations and reporting of unexpected side effects is mandatory. Although these investigations certainly are particularly important the first years after introduction, it is essential to keep having a critical eye to the development of late side effects. As an example, if a drug increases the risk for malignancy, the time from the first exposure to cancer can take years. Moreover, many of the old drugs still used today have in fact escaped the rigorous drug development process discussed above. Interestingly, some of these old pharmaceuticals would most definitely not have been approved, should they have been introduced today.

1.4 CAUSALITY AND ASSOCIATION

An association (in statistical terms usually referred to as correlation) can be defined as a state in which two variables (for example *A* and *B*) occur together more or less often than expected by chance. If an association is

found, it can be tempting and easy to jump to premature conclusions that there is a direct link (i.e. causality) between *A* and *B*. Although this may be the case, one must remain cautious as a correlation in statistical terms is not automatically a causation. Within science in general, and medicine in particular, probably the most desirable overall research aim is proving causality between an exposure and an outcome. Nevertheless, confirming causality within medicine is usually incredibly cumbersome and requires careful consideration and evaluation before it can be widely accepted.

Let me give you a well-known example. Nowadays, it is common knowledge that smoking, to a large extent, causes lung cancer. However, I think we have all seen commercials when even doctors promoted smoking before the risks had been unraveled. Even though concern about a possible link had been raised earlier, the first report that had significant influence was published in 1950. In this paper, Doll and Hill published a case-control investigation that clearly linked smoking (*the exposure*) with lung cancer (*the outcome*).[7] Nevertheless, this paper only demonstrated a significant epidemiologic association, and the carcinogen (i.e. the specific compounds that caused cancer) remained obscure.

In an influential paper in 1965, Bradford Hill (the same person as above), set out nine epidemiological viewpoints to determine whether an observed association is causal (Table 1). All of these viewpoints should

be taken into account before considering causation.[8,9] Furthermore, a reasoning around these viewpoints, in particular the criteria for *temporality* as well as *biological gradient* (i.e. dose-response correlation) is usually needed when associations are published in medical epidemiology. Epidemiological investigations can, intrinsically, never demonstrate causality, but merely point at associations. Associations found by epidemiology require a biological explanation and context. Sometimes, as Hill also pointed out,

this is futile. Therefore, the criteria should be regarded as flexible guidelines rather than a rigid checklist. Importantly, the fact that epidemiology as well as science overall have developed significantly over the past 50 years, has brought a wider range of complexity in making associations within medicine. As an example, a better understanding of molecular biology, toxicology as well as genetics has made us comprehend the convolution behind human disease.[10]

TABLE 1 The Bradford Hill epidemiological viewpoints of causality.

1	Strengh	Statistically strong association.
2	Consistency	Has the association been repeated by other research groups?
3	Specificity	How generalizable is the association?
4	Temporality	The outcome has to occur after the exposure.
5	Biological gradient	Dose-response.
6	Plausibility	Is there a plausible mechanism between the exposure and the outcome?
7	Coherence	Coherence between epidemiological and laboratory findings.
8	Experiment	Do experimental data support the association?
9	Analogy	The effect of similar factors may be considered.

1.5 THE BACKGROUND TO THE SCIENTIFIC QUESTION

A cold Tuesday in 2014, we had one of our weekly patient conferences at our Department of Dermatology at Sahlgrenska University Hospital in Gothenburg. At these conferences complex patient cases are often presented. We meet up and colleagues will give a brief introduction to their clinical question(s). Afterwards, all dermatologists examine the patients and, finally, we discuss the cases and usually end up with consensus conclusions as well as treatment plans. One of the patients demonstrated that day was a male in his 40s. He had severe psoriasis (Pso) and was evaluated for systemic treatment due to the gravity of his disease. The reason why he was brought up for discussion was because he had a history of cutaneous malignant melanoma (CMM) that was successfully removed a couple of years back. Clinical controls thereafter had been unremarkable and no disease recurrence had been observed. Several suggestions were given including methotrexate (MTX), which usually is the first systemic drug considered for treatment of moderate to severe Pso. Nevertheless, one of the senior colleagues recalled and referred to a study that was integrated in the Swedish guidelines for systemic treatment of Pso published in 2011.[11] The colleague informed us that a history of CMM most likely was a contraindication for MTX treatment. In conclusion, the patient was recommended an alternative therapy, other than MTX which, overall, is a particularly useful drug in the dermatological armamentarium. This specific clinical

decision attracted my attention as I thought it was a significant clinical crossroads. After the conference, I withdrew to my corner and read the publication which was referred to earlier. I was somewhat surprised to see that just one small Australian study[12] had such a profound impact on our clinical decision that day. Moreover, the investigation was well-cited (i.e. more than 100 citations on Google Scholar) and it seemed like the results had been widely distributed. I thought to myself that this was an important gap in research that would be interesting to investigate further. Some ideas are harder to let go of than others and this idea grew until I finally adopted it as my research question.

Does MTX treatment increase the risk of CMM?
As with all other research questions, several others emerged along the way and finally I ended up with many questions as well as side-tracks. As a consequence, the idea to include this as a part of my Ph.D. project eventually evolved. Along the way I have learned much, and it is my hope and wish to take you along an interesting journey when sharing my results.

1.6 LAYOUT

As this is a compilation thesis, brought together by six papers presented at the end, it is my intention to give you a brief background to important topics in these introductory chapters. Throughout the text, it is my aim to avoid complicated language.

I have previously introduced the concept of risk as well as association and causation. I

have also given a short introduction to the hallmarks of the drug development process and authorities involved in pharmacovigilance.

The upcoming section starts with an overview of CMM, which is the outcome of interest for all papers. Thereafter, I introduce MTX which acts as the drug of interest in *Papers I–V*.

The final investigation revolves around dermoscopic findings in melanomas and therefore I present a background to dermoscopy, which is a useful tool in clinical Dermatology. Before moving on to the methods section, I introduce the field of epidemiology and present different types of epidemiological investigations.

In the methods and results sections, I present how the investigations were conducted and the main results.

In the discussion, I examine methodological considerations including limitations and strengths of the investigations as well as a general discussion of the findings.

Finally, a chapter regarding future perspectives will give you a road map as to where I am heading next. In this section, I present some reflections made while working on this project. Moreover, a paragraph is dedicated to resuming what I have learned while struggling with the research issues. After all, this dissertation only marks the beginning of my academic career.

1.7 CUTANEOUS MALIGNANT MELANOMA

Melanoma is a cancer that originates from melanocytes, the cells that form our pigment - melanin. Melanocytes are derived from the ectoderm (neural crest) and exist in various body linings, including the uvea, intestines, mucosal linings and central nervous system. As a consequence, melanomas can occur wherever there are melanocytes (i.e. in several body sites). However, as melanomas arising in tissues, other than the skin, are exceedingly rare, when talking about melanoma in everyday clinical practice, physicians generally refer to the cutaneous form of melanoma (*cutaneous malignant melanoma - CMM*).

In normal skin, melanocytes reside in the basal layer of the epidermis, where they produce and distribute pigment to surrounding keratinocytes as demonstrated in Figures 1a and 1b. Interestingly, and perhaps contrary to belief, the density of melanocytes is roughly the same, regardless of the color of your skin. However, a dark-skinned individual has a more active melanocytic population as well as a dominance of eumelanin (brown/black melanin and a different size and shape of melanosomes), resulting in increased pigmentation and, hence, darker skin.[13]
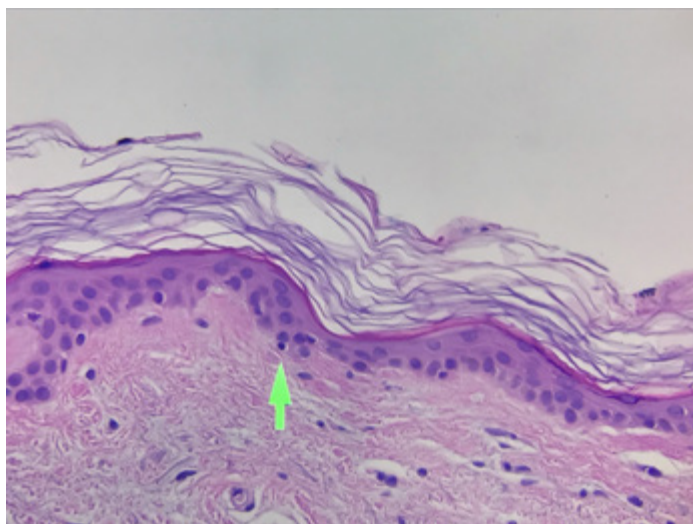
FIGURE 1a Histological slide of skin *(H&E)*. The green arrow points at one melanocyte residing in the basal layer of epidermis.
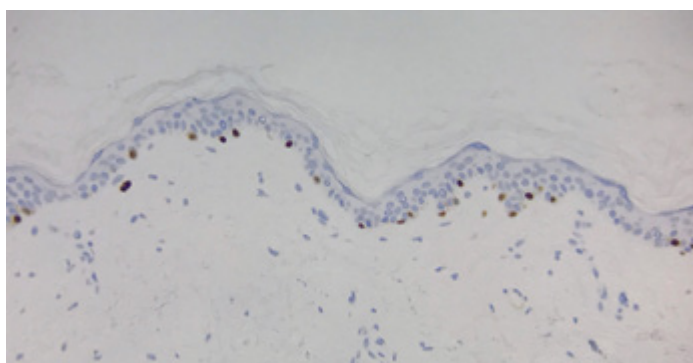

FIGURE 1b Immunohistochemistry slide of skin *(SOX-10)*. The stained cells *(in brown)* represent melanocytes residing in the basal layer of epidermis.

CMM is one of the most frequent cancer types in Sweden (the fifth most common cancer type among men and women). In 2017, 4075 new cases of invasive melanomas were reported (in 2053 men and 1880 women). When *in situ* melanomas (i.e non-invasive melanomas) are included, 8984 cases were observed in 2017.[14] As demonstrated in Figure 2, a dramatic incidence increase has been observed since 1982 in various populations. The incidence of CMM has increased approximately 3 % per year in the time period 1982 to 2011, and is expected to continue to increase until 2022.[15,16] Globally, CMM causes approximately 55,500 deaths annually. In Sweden, over 500 deaths occur due to CMM every year and the number of melanoma deaths per 100,000 person-years was 5.7 in men and 4.4 in women (in 2017). As indicated previously, men have a slightly higher risk for melanoma mortality compared to women. CMM is costly for society, in particular for metastasized disease that usually require expensive chemotherapeutical agents or immunotherapy. This means that prevention programs are potentially cost-effective or even cost-saving.[17]
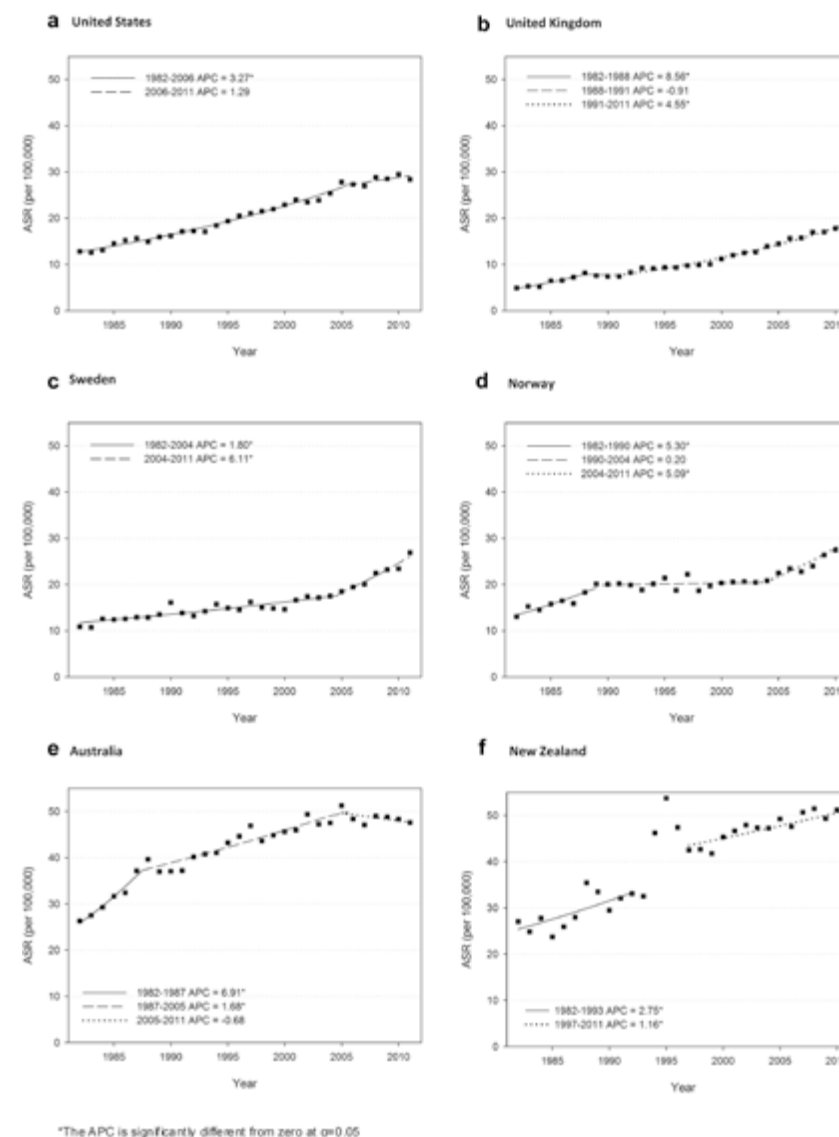

FIGURE 2 Incidence of melanomas in different populations from Whiteman *et al.*[15], *with permission from Elsevier.* Abbreviations; APC, annual percentage change; ASR, age standardized rate (US 2000).

Even though most CMMs are diagnosed clinically, it is the pathologist that confirms the definitive diagnosis. When a CMM is diagnosed, the responsible pathologist as well as the responsible physician file cancer reports that are registered at the Swedish Cancer Registry. Invasive melanomas are subcategorized histopathologically and the four most common types are: superficial spreading melanoma; nodular melanoma; lentigo maligna melanoma (that more frequently occur on facial skin), and acro-lentiginous melanomas (that occur on the palms and soles). In addition to these, several rare variants exist but will not be discussed in this setting. Superficial spreading melanoma is the most common subtype of CMM. All CMMs except possibly nodular melanoma initially display a horizontal growth phase. After a time period, which may differ from patient to patient, melanomas exert a vertical growth phase. The distance from the granular cell layer of the epidermis to the deepest melanocytes that constitute the melanoma is referred to as the Breslow depth (Figure 3) and is measured in millimeters (mm).[18] Remarkably, even though melanoma research including several genetic biomarkers have emerged, the Breslow depth is still the single best predictor of prognosis.

The Breslow depth and coexistence of ulceration, which is another histopathological finding, defines the T score in the TNM classification of melanomas according to the Union for International Cancer Control. The letter N in the acronym refers to the presence of nodal disease (i.e. metastasis to lymph nodes), and M refers to the presence or absence of distant metastases.[19] The disease burden of melanoma is classified in a clinical and pathological staging system ranging from Stage 0 (only *in situ* melanoma) to Stage IV (any presence of distant metastases). Stage 0, I and II comprise localized disease, whereas Stage III and IV implies that there is a metastasis in the regional lymph nodes or distant metastases, respectively. A detailed overview of the staging of melanoma disease is presented in Figure 4.
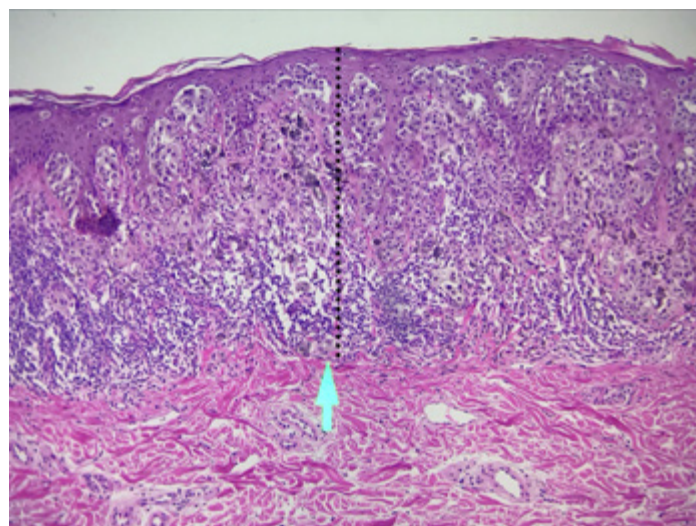

FIGURE 3 A histological section of a superficial spreading melanoma (pT1a; Breslow 0.65 mm). The green arrow points at the deepest atypical dermal melanocytes. The Breslow depth is illustrated by the dotted line.

FIGURE 4 Staging of melanoma, Used with the permission of the American College of Surgeons. Amin, M.B., Edge, S.B., Greene, F.L., *et al.* (Eds.) AJCC Cancer Staging Manual. 8th Ed. Springer New York, 2017.

In Sweden, patients with melanomas with no sign of metastasis at diagnosis are usually followed by dermatologists, whereas metastasized disease is followed by general surgeons and/or oncologists.

To optimize and standardize care and to minimize regional differences in management, patients with clinically suspected CMMs are taken care of in accordance to national guidelines.[20] Selected cases are also discussed at multidisciplinary conferences to individualize and optimize the treatment and care for particular patients.

For the context of this thesis, it is central to present CMM risk factors. In general risk factors are categorized as modifiable or environmental (the patient can influence the risk factor) and non-modifiable or phenotypic (the patient is unable to influence the risk factor). Well-known and established risk factors for CMM include: ultraviolet (UV) radiation by sun exposure and subsequent sunburns; indoor tanning; the presence of a large number of melanocytic naevi; multiple large naevi with a diameter > 5mm; a personal history of CMM and or non-melanoma skin cancer (NMSC); a family history of CMM; a phenotypic characteristic including blond or red hair, blue eyes, and/or fair skin type with a tendency to freckle, and a high socioeconomic status.[21,22] When interpreting the results and subsequent conclusions of this thesis, it is instrumental to remember that only a few of these risk factors can be accounted for in a retrospective registry-based analysis.

1.8 MELANOMA AND IMMUNOSUPPRESSION

Immunosuppression is usually defined as the complete or partial suppression of the immune response of a patient. As one can imagine, immunosuppression may have various reasons including rare genetic disorders, infections such as HIV, malignancies including lymphoproliferative diseases (LPD), radiotherapy, chemotherapy and immunosuppressive drugs. The immune system does not only protect against infections but also protects the host from cancer cells.[23] CMMs have a more unfavorable prognosis in patients in a clinical setting of immunosuppression.[24]

A well-investigated patient group that constantly needs to be in an immunosuppressive state are organ transplant recipients (OTRs). In order to avoid rejection of the transplanted organ, this patient group requires immunosuppressive drugs with different modes of action. Significantly, this immunocompromised group is particularly prone to develop cutaneous malignancies including CMM. As a consequence, to address this issue, OTRs are invited to regular follow-ups at most Dermatology departments. In a systematic meta-analysis including 20 cohort studies and 367,477 patients, OTRs had a pooled relative risk for CMM of 2.71 (95% CI [confidence interval] 2.23–3.30) compared to the background population.[25] This number corresponds well with results from a nationwide Swedish retrospective investigation, including 10,476 OTRs in the time period of 1970 to 2008.[26] In this cohort, 52 cases of CMM were diagnosed among 51

patients, standardized incidence ratio (SIR) of 2.2 (95% CI 1.7-2.9).

In another publication, including CMMs arising in OTRs in the time period 1984-2008, 49 cases were observed and re-examined. CMMs among the OTRs were more advanced compared to the general population and the melanoma-specific mortality was increased.[27]

Clearly, CMM and the immune system have attracted a lot of attention during the past decade. It is not an exaggeration that the incredible development of immunotherapy, which is a new type of anti-cancer drugs that stimulate the cancer-specific immune system, has revolutionized treatment for metastasized melanoma disease.[28-30] Interestingly, CMMs infiltrated by lymphocytes (tumor-infiltrating lymphocytes) have a more favorable prognosis compared to individuals without the presence of these cells.[31]

In an American publication, cancer incidence among HIV-infected patients were calculated and compared to the general population yielding a SIR for CMM of 2.6 (95% CI 1.9-3.6) for HIV infection.[32] Interestingly, a Danish nationwide cohort study could not demonstrate an increased risk for CMM among HIV-infected individuals (n=4280) compared to sex- and age-matched cohort (n=21,399) with an incidence rate ratio (IRR) of 0.60 (95% CI 0.28–1.31). However, the authors concluded that due to few events of CMM in the cohort (n=7) solid conclusions could not be made.[33]

Although data is scarce, immunosuppressive drugs used among non-OTRs have been linked to CMM. Dillon *et al.* reported two patients with myasthenia gravis that were treated with azathioprine. Both individuals developed stage IV melanoma. Nevertheless, both tumors regressed upon withdrawal of the medication.[34] Although this type of anecdotal reporting in case reports is important, investigations of immunosuppression and CMM is intrinsically difficult to investigate as the group is heterogenous and relatively rare.

1.9 MELANOMA AND OTHER DRUGS

Perhaps not surprisingly, researchers have investigated whether certain drugs may influence the risk or the disease progression of skin cancer in general and CMM in particular.[35] The results of such investigations are especially important as they may influence prescription pattern for this specific patient group.

Interestingly, a common phosphodiesterase type 5 inhibitor (PDE5i), sildenafil, which is prescribed for erectile dysfunction in men, has been linked to an increased risk for CMM.[36] This finding was reproduced in a Swedish nested case-control investigation. However, as there was no increased risk among men with multiple filled prescriptions, the authors raised the question whether the association was causal.[37] Having two investigations with somewhat conflicting results, another retrospective investigation was conducted in the United Kingdom (UK) including approximately

150,000 men. Only a weak association between exposure to a PDE5i was found. However, the authors suggested that the association was non-causal and was explained by greater sun exposure among PDE5i users.[38] In a recent meta-analysis, including all available investigations to date, a statistically significant increase of CMM was observed in patients who were prescribed with PDE5i. However, there was no increased risk for patients with a high use compared to patients with a low use.[39]

In nationwide Danish investigations, the commonly used antihypertensive drug hydrochlorothiazide was recently linked to an increased risk for squamous cell carcinoma, lip cancer as well as CMM.[40-42] The rationale behind the association is likely due to the fact that hydrochlorothiazide has photosensitizing effects and in combination with UVA induces DNA damage in cells of the skin.[43]

Beta blockers have been investigated in melanoma progression, and experiments conducted in mice demonstrated that propranolol (a non-selective beta blocker) slowed melanoma development in mice transplanted with human melanoma cells.[44] Nevertheless, in a retrospective investigation conducted in the UK, post-diagnosis beta blocker medication among CMM patients did not reduce the risk for melanoma death nor all-cause mortality.[45]

The anti-diabetic drug metformin has

demonstrated promising anti-melanoma properties in experiments. However, in an open-label clinical trial, no effect on metastasized disease was observed.[46]

Association between nonsteroidal anti-inflammatory drugs (including acetylsalicylic acid) and the risk of cancer including CMM have been examined in multiple investigations. There are, however, conflicting data as to whether this group of drugs influences the risk for CMM.[47-50] Nevertheless, in a recent retrospective investigation, post-diagnosis acetylsalicylic acid was associated with a longer overall survival in patients with CMM in stages II and III.[51]

As TNF (Tumor Necrosis Factor) is a key cytokine which orchestrates an appropriate immune response, the risk of cancer in general and CMM specifically after TNF inhibitor (TNFi) treatment has been debated ever since the introduction of this drug group some 20 years ago. Various investigations have demonstrated somewhat conflicting results. However, in a meta-analysis, including data from nine European countries, no significant risk increase for CMM was observed after exposure to TNFi treatment in rheumatoid arthritis (RA) patients.[52] Nevertheless, as data are conflicting, it is not excluded that there may be an increased risk for CMM among patients treated with TNFi.[53] In a recent Swedish investigation, including all RA patients that had been treated with TNFi as the first or second biological drug, no increased risk for a first invasive CMM was observed

compared to a cohort of patients treated with conventional systemic disease-modifying antirheumatic drugs (DMARDs).[54]

In this context, the cholesterol-lowering drug group statins, which is one of the most frequently prescribed drug groups in USA, has attracted attention since it has demonstrated effect against CMM progression in cell cultures and halted clinically evident metastases in mice.[55] Nevertheless,

the *in vitro* and *in vivo* effects against CMM has not been observed in epidemiological investigations.[56,57]

In summary, as you can see in this section, several frequently used drugs, including immunosuppressive agents, have been under scrutiny in the context of CMM. Future investigations will help clarify possible associations and, potentially, a causal link between exposure to these drugs and CMM.

defects, several countries have introduced fortification of folic acid in flour. However, due to fear of increasing cancer incidence as well as a worse cancer prognosis, some countries avoid folic acid fortification. In a comprehensive meta-analysis including 13 randomized placebo controlled clinical trials and 50,000 patients, no evidence of an increased cancer risk was observed among patients who were randomized to folic acid treatment. Moreover, in subgroup analysis, no risk increase of CMM was observed.[61]

Methotrexate – pharmacokinetics

Pharmacokinetics refers to how a drug is metabolized and distributed in the body. MTX is usually taken as tablets (i.e. per oral use), but can also be administered subcutaneously and intramuscularly (rare). Interestingly, there is substantial interindividual pharmacokinetic variability when MTX is administered. This is because of differences in intestinal absorption, renal elimination as well as differences in pharmaco-genetics.[62-64]

When taken orally, MTX is absorbed through the reduced folate carrier in the proximal part of the jejunum. The bioavailability (i.e. the proportion that reaches the site of action) is generally 70-80%. The absorption is not affected if food is taken with the drug. After entering the blood stream, MTX is partially metabolized in the liver where an inactive metabolite, 7-hydroxymethotrexate, is formed. After liver metabolism, MTX binds to albumin (35-50% affinity), and is later transported intracellularly by the reduced folate carrier. In the intracellular compartment, MTX is transformed to MTX-polyglutamate, which is the active compound for exerting anti-inflammatory action. MTX is eliminated mainly through renal clearance.[65]

Methotrexate – modes of action

MTX is a folate antimetabolite that has a similar chemical structure to folic acid (Figure 5) and irreversibly binds to and inhibits dihydrofolate reductase.
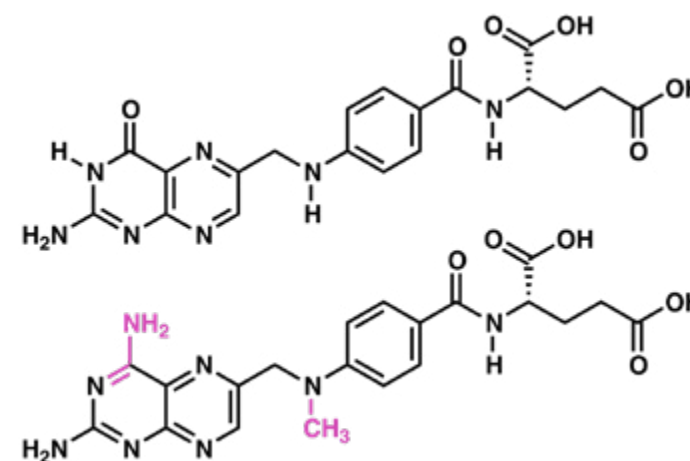
FIGURE 5 Figure demonstrating the chemical similarity between folic acid (top) and methotrexate (bottom).

1.10 METHOTREXATE – AN OVERVIEW
Folic acid

Folic acid, also known as vitamin B9, is a water-soluble and essential vitamin. Since the human body cannot synthesize folates, dietary supplementation is crucial. Examples of foods rich in folates are dark green vegetables such as broccoli, spinach and beans. Folic acid is required for DNA and RNA synthesis and amino acid metabolism, making it essential for cell division. Folate is an umbrella term used to denote the group of chemical compounds with the same vitamin activity. Therefore, the term includes both natural folates as well as folic acid. The difference

between folate obtained by food and folic acid is important, because food folate is only about half as available as folic acid consumed on an empty stomach.[58] An advantage with folic acid is that it is chemically more stable than naturally occurring folates making it appropriate to use in food fortification and in vitamin supplements. Folic acid is instrumental during pregnancy as deficiencies in the vitamin increase the risk for neural tube defects.[59] Therefore, all women who intend to be pregnant are recommended the vitamin during conception until gestational week 12.[60]

To further decrease the risk for neural tube

This binding prevents the formation of reduced folates, and thymidylate synthetase which results in inhibition of purine and thymidylic acid synthesis. In plain language, this means that MTX interferes with DNA synthesis, repair, and cellular replication. MTX is cell cycle-specific for the synthesis phase of the cell cycle resulting in cell arrest in the growth phase. As a consequence, active and proliferative tissues are more susceptible to the effects of the drug.

The antifolate mechanism of action explains the antineoplastic and chemotherapeutic nature of MTX in cancer, where a high-dose regimen is used. However, the antifolate properties is likely not the only mechanism of action for autoimmune and inflammatory diseases such as RA, Pso and psoriatic arthritis (PsoA). Indirect evidence of this is that MTX still exert its function even when folic acid is administered. In experiments conducted *in vivo* and *in vitro*, MTX has been shown to influence several pathways of the inflammatory response such as inducing apoptosis, reducing neutrophil chemotaxis and inhibition of neo-vascularization.[66]

In a low-dose setting, MTX inhibits AICAR transformylase which is an enzyme required for *de novo* purine synthesis.[67] This inhibition results in an accumulation of AICAR which inhibits the degradation of adenosine. Accumulation of adenosine, which is an important anti-inflammatory mediator, is most likely the key anti-inflammatory mode of action.[68] Nevertheless, even when adenosine is blocked, MTX exerts an

anti-inflammatory effect suggesting more anti-inflammatory roles including inhibition of polyamine synthesis and an inhibition of monocyte recruitment.[69]

Moreover, other mechanisms of action have been discovered and MTX likely exerts several different pharmacodynamic effects, still unknown to this day. As different inflammatory and autoimmune diseases do not share the same pathophysiology, but still respond well to MTX, it is likely that different modes of action play a more pivotal role in different diseases. In RA, several different pathways and functions have been suggested and were presented in a comprehensive review by Wessels *et al.*[70] Recently, MTX was demonstrated to evoke regulatory T-cells in Pso.[71] Moreover, MTX increases the intrinsic apoptosis in proliferating keratinocytes in psoriatic skin.[72] At the end of the day, the definitive mode of action remains elusive and more suggested modes of action in various inflammatory diseases are expected. Finally, MTX might even exert different effects on different individuals.[73] A selection of suggested modes of action is presented in Figure 6.
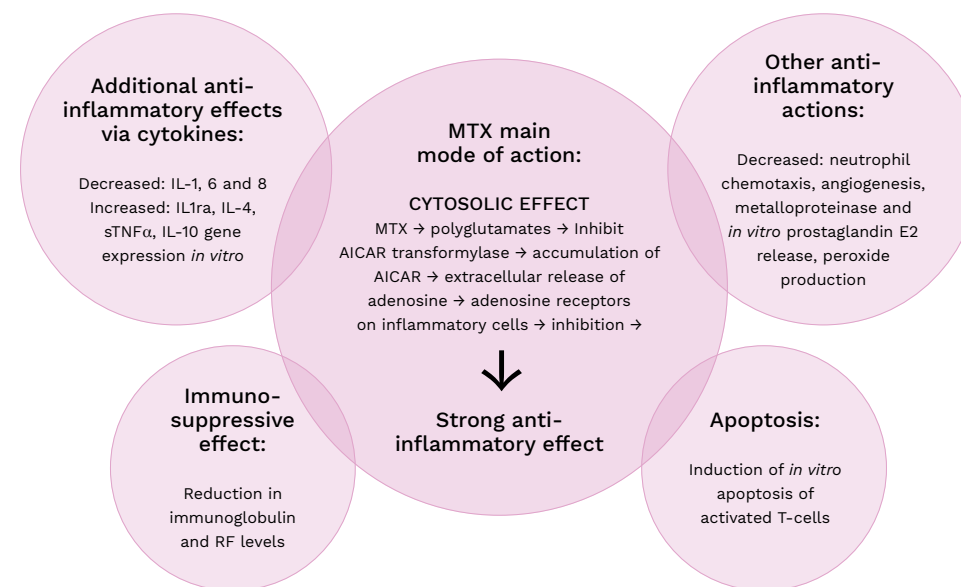
FIGURE 6 A selection of MTX - modes of action. Adapted from Malaviya *et al.*[74], Abbreviations; IL, Interleukin; IL1ra, Interleukin-1 receptor antagonist; sTNFα, soluble Tumor Necrosis Factor α; MTX, methotrexate; AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide; RF, Rheumatoid factor.

Different dose regimens of methotrexate

Depending on the clinical situation, MTX is administered in different dose regimens (Table 2). When MTX is used as a chemotherapeutical a high dose of MTX (HD-MTX) is administered whereas a low dose (LD-MTX) is used for inflammatory and autoimmune disorders.

As demonstrated in the table, doses used in HD-MTX are exponentially higher than LD-MTX. As an example, the low dose regimen rarely exceeds 25 mg/week, whereas grams of MTX can be administered in an antineoplastic setting. The fact that there are significant differences in dose and likely different main modes of action, LD-MTX

TABLE 2 The different dose treatment regimens of MTX. The three intervals depend on the dose per body surface area (m²). The table is adapted from UpToDate.[75]

High-dose MTX:	doses ≥ 500 mg/m²
Intermediate dose of MTX:	doses between 50 and 500 mg/m²
Low-dose MTX:	< 50 mg/m²

and HD-MTX should therefore be regarded as two different therapeutic agents in practical terms.[74] As this thesis only revolves around MTX used in a low dose setting, the high dose regimen will not be reviewed extensively. Nevertheless, as you will see in the next paragraph, this was indeed how MTX was firstly introduced.

History

Folic acid was first crystalized and isolated in 1945.[76] Soon after the discovery, the compound was tested in children with acute leukemia, which, at that time, had no curative nor effective treatment. Upon supplementation of folic acid to these patients an acceleration phenomenon on the leukemic process was observed.[77] Oppositely, a diet deficient in folates slowed down the leukemic process. These observations suggested that folate antagonists might be an appealing treatment option for patients with acute leukemia, which is characterized by a high cell turnover. Aminopterin was the first antagonist to be developed and was first clinically tested in children with acute leukemia. In a pioneering paper by Farber *et al.* temporary disease remissions among these children were observed.[78] This pivotal paper marked the beginning of modern chemotherapeutics.[79] In this context, the scientist behind the development of aminopterin as well as MTX, the American-Indian biochemist Yellapragada SubbaRow (Figure 7), deserves special admiration. Earlier in his career, SubbaRow had described the function of adenosine triphosphate and phosphocreatine. MTX, which initially was

named amethopterin, was subsequently the drug of choice due to less toxicity as well as easier manufacture process.

Having antimetabolite effects on tissues with high cell turnover lead to testing of aminopterin in RA and Pso. As early as 1951, Gubner *et al.* presented a case-series

FIGURE 7 Yellapragada SubbaRow, the man behind the discovery of aminopterin and methotrexate.

where a low dose regimen of aminopterin was evaluated. Seven patients with RA and one patient with acute rheumatic fever were treated. Good clinical response in the arthritis process was observed for these patients. Interestingly, one patient in the RA group had concurrent Pso and experienced clinical response on psoriatic lesions. Aminopterin was subsequently tested in five additional

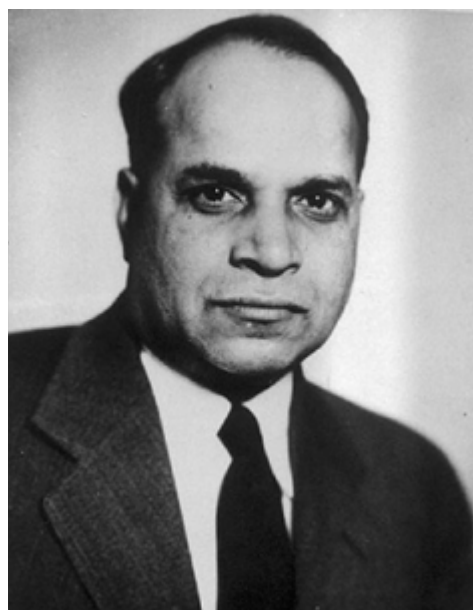patients with long-standing Pso complicated with arthritis and three patients with uncomplicated Pso as well as one with chronic dermatitis. The psoriatic skin lesions responded well to the therapy.[80,81] Having said that, within the Rheumatology community, the findings did not significantly influence the treatment patterns noteworthily, because cortisone was still the drug of choice for treating RA. Interestingly, at his clinic in Spokane, Washington, Hoffmeister started treating RA patients with intramuscular MTX in 1967. His work was initially published as an abstract[82], but was not well-received at that time. Nevertheless, he continued his treatment regime, but it was only in 1983 that he published a landmark paper on MTX for treatment of RA. Overall, 78 RA patients that had inadequate control after conventional treatment were included. Among these, 45 patients experienced a marked improvement or complete remission.[83] In the same time period, Willkens *et al.* published data with similar results.[84,85] These publications paved the way for several randomized clinical trials of MTX in RA conducted in the mid 1980s and beginning of the 1990s.[86-91] Therefore, for RA, the documentation prior to a broader acceptance was acceptable. However, surprisingly few clinical trials have evaluated MTX for Pso. In 1958, Edmunson *et al.* presented a case series including 62 Pso patients treated with aminopterin (n=32), MTX (n=17) or both (n=13) and good clinical responses were observed.[92] Perhaps, as Pso did not have any other systemic treatment during the 1960s MTX was more colloquially used

even without the guide of clinical trials. By the end of the 1960s, it was considered standard dermatological practice.[93] It was only in 1972 that the first clinical guidelines were published by Roenigk *et al.*[94] Nevertheless, at that time, it was expected that 50,000 had already filled a prescription of the drug.[84]

Formal approvals and importance

MTX received formal U.S. Food and Drug Administration approval for treatment of Pso in 1971 and in 1988 for the treatment of RA. In Sweden, MTX was first approved in 1964. Importantly, MTX is included in the WHO (World Health Organization) Model List of Essential Medicines and is listed as an essential drug among cytotoxic and adjuvant medicines, as well as among DMARDs.[95]

Contemporary use in the clinic

In Sweden, LD-MTX has a formal indication for RA, PsoA, Pso, juvenile idiopathic arthritis as well as Crohn's disease.[96] All pharmaceutical drugs that are available on the market receive a designated Anatomical Therapeutic Chemical (ATC) code. The lists are managed and organized by the WHO and they do not differ between countries.[97] As MTX have both antineoplastic as well as antimetabolic properties, the drug has two designated ATC-codes within the antineoplastic (*L01BA01*) and immunomodulating agents (*L04AX03*) groups. In an outpatient setting (i.e. MTX in a low-dose regimen) the code *L04AX03* is almost exclusively used. Interestingly, in 2016, MTX was the 152nd most prescribed drug in USA with more than 4.2 million prescriptions.[98] In Sweden,

the number of patients over 20 years of age that were filled a MTX prescription increased from ≈ 41,000 in 2007 to ≈ 62,000 in 2017 (38 % men and 62 % women). Another way to describe the frequency is the number of users per 1000 people in the population. Among individuals over 20 years, 8.07 patients/1000 individuals were filled MTX prescriptions in 2017. Conveniently, these statistics on a population level are easily available online for the general public.[99] Importantly, for a health care system that is financed by tax payers, MTX is an inexpensive drug. In comparison to pharmaceutical drugs developed today with pending patents, the cost is negligible. When follow-up visits and blood test monitoring are excluded, the weekly cost for a patient that is on a dose of 15 mg/week *per os* (a common maintenance dose), is only approximately 6 SEK. When administered subcutaneously, the corresponding cost would be approximately 150 SEK. In contrast, the cost for maintenance therapy with a TNFi is roughly 1000 SEK/week.[96]

In Sweden, Pso is the most common diagnosis that prompts prescription of MTX among dermatologists. Within Rheumatology, RA and PsoA are the two most common diagnoses that are treated with the drug. Although no other formal and approved indications exist, MTX is used in a range of dermatological conditions including proliferative dermatoses, immune-bullous dermatoses, autoimmune connective tissue diseases, vasculitis, dermatitis as well as numerous miscellaneous diseases.[75,100] Even

though different treatment dosing schemes are used, MTX is most commonly administered once per week. Having effect on actively dividing cell linings help explain the most common side effects of MTX which are: stomatitis, dyspepsia, anorexia, nausea, vomiting and abdominal pain. To modulate the side effects, folic acid is usually administered 24 h after MTX administration. Alternatively, folic acid is prescribed all days but the day of MTX intake. If a patient experiences some minor side effects, but has a good clinical response, MTX can be administered subcutaneously.

High-dose methotrexate and risk for malignancy

Even though *Papers I-V* of this thesis revolve around LD-MTX, a paragraph about HD-MTX and the risk for secondary malignancies is important as it will shed light on the research question.

As mentioned above, MTX was indeed first developed as an antineoplastic chemotherapeutical drug, and is still used clinically for specific malignancies including osteosarcoma and primary central nervous system lymphoma. In an oncologic setting, MTX is administered parenterally in doses that often are log orders higher than in the low-dose setting. Moreover, MTX is almost exclusively administered with other concurrent antineoplastic drugs (i.e. combination therapy). Needless to say, this complicates investigations of the risk for a second neoplasm induced by MTX alone. However, an exception to this are gestational trophoblastic

tumors (GTT) which is a rare form of tumor arising in the trophoblast unit of the placenta. For this patient group, MTX has been used as a single chemotherapeutic treatment in the majority of cases since the introduction in 1956.[101] Moreover, the overall prognosis for GTT is excellent which allow a long-term follow-up.

In the UK, the care, treatment and follow-up of patients with GTT are arranged in two centers which permit a unique opportunity to obtain long-term outcome data on a large scale for patients. Ruslin *et al.* examined the risk of developing a second neoplasm after treatment with cytotoxic chemotherapy against choriocarcinoma or invasive mole. In a first investigation including 457 women (3522 patient-years, mean observation period 7.8 years) there was no support for an increased risk of a second neoplasm (2 cases of malignancy observed whereas 3.5 were expected).[102] A follow-up study, including more patients and a longer observation period, demonstrated that combination chemotherapy for GTT tumors increased the risk of secondary malignancy. However, no increased risk was seen for women who received MTX as the only chemotherapeutic therapy with a relative risk of 1.3 (95% CI 0.6-2.1).[103] The most recent follow-up study was published in 2015 and included 871 women who had been treated with a MTX-Folic acid regimen without any combination therapy (15,499 person-years and an average follow-up of 17.8 years). Here, 26 second neoplasms were observed whereas 35.74 were expected (SIR 0,7; 95% CI 0.5-1.1). Importantly, among this

group of patients, 4 cases of invasive melanoma were observed whereas only 1.74 were expected (SIR 2.3; 95% CI 0.9-6.1). *In situ* melanomas were not included in the calculation.[104]

Interestingly, but from a dermatological viewpoint, anecdotally, HD-MTX as monotherapy has been tried for the treatment of patients with multiple basal cell carcinomas. Tumors of these patients became hemorrhagic and superficially necrotic in response to drug therapy. Most tumors were reduced in size but none disappeared entirely. Eradication of all tumors was not achieved in any patient.[105]

Treatment for metastasized melanoma has historically been a tremendous challenge and several chemotherapeutics have been tested with inadequate results. Therefore, perhaps not surprisingly, HD-MTX has indeed been evaluated for the treatment of advanced metastasized melanoma disease.[106-111] While clinical response was observed in some patients[106], other investigations indicated more gloomy results.[107] Ultimately, it was concluded that HD-MTX had no role in the treatment of advanced melanoma disease.[110] This might be a consequence of a natural resistance to MTX observed in human melanoma cell lines.[112]

Low-dose methotrexate and risk for malignancy

An immunosuppressive and anti-inflammatory drug such as MTX that has been used clinically for decades has been under

scrutiny regarding risk for the development of cancer. Nevertheless, MTX has clearly not been the focus of most contemporary pharmacoepidemiologic studies of cancer. Although frequently used as an immunosuppressive treatment in patients with chronic autoimmune and inflammatory diseases, it is still controversial whether MTX increases the overall risk of malignancies.[113]

Buchbinder *et al.*

As the idea of this thesis in fact emerged after reading this single investigation, it deserves particular attention. In fact, prior to our investigations, it was the only study that had investigated the risk of CMM in patients treated with MTX. Additionally, the text below will hopefully give you an understanding and roadmap as to how we addressed our research question.

Buchbinder *et al.*[12] performed a retrospective cohort investigation, including 458 patients with RA. The average follow-up for each patient was 9.3 years and the analysis was performed on 4145 person-years. The patients were followed in an outpatient setting by six rheumatologists in the Melbourne area. A total of 64 cases of cancer were reported during the follow-up period whereas 42.6 were expected (SIR 1.5; 95% CI 1.2–1.9). Using incidence rates from the background Australian population, 2.3 cases of invasive melanoma were expected, but in the cohort 7 cases were observed (SIR 3.0; 95% CI 1.2–6.2) (Table 3).

TABLE 3 Standardized incidence ratios for malignancy overall and for specific malignancies in a methotrexate-treated rheumatoid arthritis cohort of 458 patients. The table is adapted from Buchbinder *et al.*[12] *Printed with permission from John Wiley and sons.*

	Observed cancers, no.	Expected cancers, no.	SIR	95% CI
Overall	64	42.6	1.5	1.2–1.9
Lung	14	4.9	2.9	1.6–4.8
Non-Hodgkin's lymphoma	8	1.6	5.1	2.2–10.0
Melanoma	7	2.3	3.0	1.2–6.2
Colorectal	6	7.4	0.8	0.3–1.8
Bladder	4	1.9	2.2	0.6–5.5
Breast	4	6.1	0.7	0.2–1.7
Liver	1	0.3	3.7	0.1–20.8
Hodgkin's lymphoma	1	0.1	8.9	0.2–49.8

Abbreviations; CI, confidence interval; SIR, standardized incidence ratio.

No control group of RA patients unexposed to MTX were examined, making it difficult to determine how much of the observed increased risk of malignancy could be attributed to MTX in the specific population. Moreover, the accumulated doses of MTX was not presented making it difficult to address a potential dose-response association. Interestingly, *in situ* melanomas were not included in the cohort. As the investigation only included RA patients in an outpatient setting among private rheumatologists, it is likely that patients with more severe disease (usually followed-up at the hospital level) were excluded. Moreover, the authors do not discuss whether only patients with health care insurance were included. If only this patient group were included, one would expect that patients with lower income and lower socioeconomic status would be excluded from the analysis. The investigation was conducted as a retrospective investigation and all possible increases of malignancy in the population was addressed. Correction for multiple comparisons was not performed and it is therefore not excluded that an increase of the frequency of melanomas was due to chance (i.e. false association may emerge if more outcomes are included in the analysis). When interpreting the results, it is important to consider the setting in which the investigation took place. The Australian population originates, to a large extent, from European ancestors (i.e. Caucasian population). However, the climate and spectrum of UV light is different. Therefore, it may be difficult to generalize the results to other populations.

Other investigations

Although infrequent, there are some other reports that have shed light on the research question and a selection of them are presented below.

Bailin *et al.* conducted an American retrospective study in Pso patients that initiated MTX therapy between 1960 and 1965. The patients were followed until 1973. Overall, 205 patients were included in the analysis. The treatment period varied from one day to nine years (average 2.7 years). The MTX doses varied from 10 mg to 25 grams. No increased risk in total incidence of internal malignancy was observed (observed 8, expected 6.8).[114]

Nyfors *et al.* conducted a retrospective study in Danish patients with severe Pso who initiated MTX therapy in the time period 1964 to 1973. The patients were followed until 1977 (median 7 years, range 5-14 years). Overall, 248 patients (128 women and 120 men with an average age of 52 years at inclusion) were included in the analysis. Using age- and sex-matched available national statistics, 22±7 cases of malignancies were expected whereas only 10 were observed. No cases of CMM were reported.[115]

Stern *et al.* conducted a case-control study, using patients that previously had been included in a study evaluating the health benefits of psoralen plus UVA treatment. History and exposure time of MTX use was obtained by a questionnaire (1261 patients included; 65% males, 35% females, age 46 ± 15 years).

Patients with cutaneous and non-cutaneous malignancies (cases) were compared to age- and sex-matched patients without such malignancies (controls). MTX exposure status was examined in the respective group and no statistical difference between the groups was observed. This investigation suggested that MTX did not increase the risk of non-cutaneous nor cutaneous malignancy in patients with severe Pso.

Alarcon et al. investigated MTX drug survival rate in a cohort of 152 American RA patients with a total observation period of 862 person-years. In a secondary analysis, the authors concluded that there was not an increased rate of cancer deaths among patients with MTX exposure (observed 4 expected 3.88, standardized mortality ratio 103; 95% CI 28 – 263).[116]

West et al. conducted a comprehensive investigation including two meta-analyses on MTX safety and efficacy measures for Pso. Within the prospective investigations included in the analysis, 11 trials specifically reported on malignancy. A weighted incidence for all malignancies of MTX-treated individuals was 1.2% (median duration 12 months range 5.5–24 months; 2465 patient-years).[117] In this large investigation, only one case of CMM was recorded in one patient on MTX.[118] Clinical trials are often limited in time and therefore long-term reporting of side effects is not included. Thus, clinical trials, albeit well-designed, do not reflect the intended use of MTX (i.e. continuous long-term use).

Although, MTX is relatively rarely included in pharmacoepidemiologic investigations, Solomon et al. conducted a comprehensive analysis of a large cohort of RA patients, from an American cohort, with respect to their treatment. In this investigation, MTX, other non-biologic as well as biologic DMARDs were compared. MTX was associated with an increased cancer risk compared to non-biologic DMARDs and TNFi. Nevertheless, too few cases of CMM were included to make relevant comparisons for that particular drug.[119]

In Sweden, a nationwide RA register exists with a good coverage. In a prospective cohort investigation conducted by Raaschou el al., the authors concluded that patients with RA who had not been treated with biological drugs are not at increased risk of invasive melanoma compared with the general population. Patients selected for TNFi treatment had a 50 % relative risk for invasive melanoma compared to RA patients not receiving TNFi.[120] Nevertheless, there were not more in situ melanomas among the patients treated with TNFi, and, moreover, there was not an increased overall risk for cancer. In a subgroup analysis, a detailed investigation of MTX exposure among the both groups was conducted and adjusted for. This, however did not change the hazard ratio (HR) associated with the use of a TNFi. This is, to the best of my knowledge, the only prior Swedish investigation that had investigated whether MTX influences the risk for CMM. The investigation was important, but only provided indirect evidence that MTX does not increase the risk for CMM.

As MTX treatment and CMM are both quite common in the population, it is expected that overlap of the two have occurred, even though this may have been due to chance. A selection of these case reports is presented below.

Jeannou et al. reported two patients with RA treated with MTX. Both patients developed CMM. The first presented with metastatic disease at diagnosis.[121] Potter et al. reported one patient with RA and MTX treatment who presented with four synchronous CMM that all were excised.[122] Wemmer et al. published a case report with a patient with generalized Pso. As topical treatment failed, MTX was introduced in 1966 (at the age of 56 years). After two years the treatment was discontinued. The patient presented again in 1971 with extensive psoriatic disease and MTX therapy was reinitiated. In January 1973, nine tumors were detected in the area of the inner thigh. The area was excised and seven primary CMMs in different stages were observed. The patient passed away in the beginning of 1975 due to melanoma brain metastasis.[123] Recently, a primary malignant melanoma of the urethra was reported in a patient with RA with a 22 year history of MTX treatment.[124]

Methotrexate-associated lymphoproliferative disorders

There have been reports of lymphoproliferative disorders (LPD) clearly linked with MTX treatment.[125] The entity is categorized as a type of iatrogenic immunodeficiency-associated LPD. Interestingly, upon discontinuation of MTX, some of these malignancies spontaneously regress. WHO has denoted this entity as MTX-associated LPD. If a patient develops LPD during MTX therapy, discontinuation of therapy and watchful waiting is suggested as spontaneous remission most frequently occurs within 4 weeks.[125]

Methotrexate and risk for non-melanoma skin cancer

In a Tasmanian cohort including patients with RA (n=345) and PsoA (n=60), exposure to MTX was associated with an increased risk for squamous cell carcinoma and basal cell carcinoma. For basal cell carcinoma, there was a trend for a dose-response relationship. Importantly, when Ciclosporin-A or D-penicillamine were added to MTX, the risk for NMSC increased.[126] Moreover, in an American cohort of RA patients, MTX exposure ≥1 year after a first NMSC increased the risk for a new NMSC.[127]

Adverse events from recent randomized controlled trials

The time for therapeutic evaluation of MTX in randomized clinical trials are over. In fact, only a very few of these trials used placebo as an active comparator. Moreover, if a placebo group was included in the trials, it never reflected intended use which is long-term treatment. This means that it is hard to evaluate safety in this setting as several adverse events may evolve years after the initiation of drugs.

In RA patients, MTX reduces the risk for cardiovascular disease.[128] Inflammation is tightly linked to atherosclerosis.[129] As a proof of concept, a double-blinded randomized placebo-controlled clinical trial demonstrated that Canakinumab (an IL-1β-antibody) lowered the risk for a recurrent cardiovascular event.[130] Bearing these results in mind, a North American investigation[131-133] was conducted including 4786 patients (median follow-up 2.3 years) with a previous myocardial infarction or multivessel coronary disease who additionally had either type 2 diabetes or metabolic syndrome. The patients were all treated with MTX, and the patients that tolerated the drug well, were randomized to continue MTX or receive placebo treatment. Contrary to Canakinumab, no protective effect for a recurrent cardiovascular event was observed in the patient group treated with MTX compared to placebo. Nevertheless, as mandatory for modern clinical trials, safety data was recorded. Interestingly, 33 patients in the MTX group developed non-basal cell skin cancer whereas this only occurred in 12 patients in the placebo group (P=0.003). The number of CMM as well as other types of skin cancers were not specifically reported, but is likely to be disclosed in future articles. In this setting, it is important to be reminded that the patients investigated above did not necessarily have RA or Pso.

As previously stated, surprisingly few prospective investigations have been conducted with respect to MTX and clinical efficacy for Pso. Importantly, a 52-week randomized prospective trial was conducted and published in 2016 investigating the clinical effect of subcutaneously administered MTX for patients with Pso. Patients randomized to placebo could be switched to MTX after week 16. Overall, 113 patients were exposed to MTX and no cases of malignancy were reported.

In vitro and animal model observations

In a preclinical setting, MTX has been evaluated for treatment of melanoma cell lines in several publications. *In vitro* experiments conducted by Nihal *et al.* concluded that MTX inhibited the viability of human melanoma cell lines and enhanced Fas/Fas-ligand expression, promoting apoptosis through the extrinsic as well as intrinsic pathway. Moreover, the combination treatment of both MTX and IFNα2b induced apoptosis to a larger extent than either compound alone.[134] In mice xenografted with human melanoma cells from melanoma patients, MTX treatment resulted in a lower number of metastasizing cells in the blood stream and a lower metastatic burden. However, no effect was seen on the growth of subcutaneous melanoma. The results may indicate that the folic acid pathway is important for metastasizing melanoma cells.[135]

BRAF inhibitors (BRAFi) constitute an important group of drugs and these are specifically used for BRAF V600E-mutated melanomas. However, a clinical problem is that melanoma is highly mutagenic and therefore can become resistant and escape the effect of BRAFi. In an *in vitro* experiment, MTX treatment in melanoma cell lines sensitized the effect of BRAFi and induced apoptosis.[136]

Barich *et al.* conducted an experiment where mice were divided into three groups (A, B and C). All 3 groups received biweekly applications of methylcholanthrene (a known human carcinogen) for 11 weeks to the shaved epidermis of the intrascapular area. Mice in group A were given standard food. Mice in group B were given MTX 6 weeks prior, during and subsequent to methylcholanthrene applications. Mice in group C were treated with MTX one week prior, during and subsequent to methylcholanthrene applications. Most cutaneous tumors developed in group B with 6 weeks of MTX exposure whereas the least number of tumors developed in group C. The authors suggested that a prolonged administration of MTX can convert an antitumor action into a co-carcinogenic action.[137]

The observations above suggest that the folic acid pathway is involved in melanoma pathogenesis. However, the detailed effects remain to be elucidated. Moreover, it is not excluded that MTX used as a high dose regimen, might have another impact of melanoma than lower doses. It should also be noted that there might be different effects depending on the context. As an example, MTX could potentially be protective against melanoma, but it might accelerate melanoma progression if a manifested disease evolves.

Results from searches performed in databases

As clear from above, despite the early introduction, MTX is still a central and frequently used drug in Rheumatology as well as Dermatology treatment armamentaria. Importantly, ever since the introduction, a lot of clinical experience has been gained on the usage of MTX. Nevertheless, within the Dermatology field, it has, to a large extent, escaped the rigorous clinical trials that are mandatory for introduction of novel pharmaceutical agents today.[138] In Sweden, as mentioned previously, the Swedish Medical Products Agency is responsible for collecting side-effect reports and I reached out to them on January 11, 2019 to make an inventory of how many malignancies in general and CMM in particular, had been reported on patients using MTX. Overall, 158 adverse events reports had been filed regarding MTX and malignancy. However, of these, somewhat surprisingly, only one (!) adverse event had ever been filed with respect to MTX and CMM.[139] In the corresponding EudraVigilance database, 147 cases of CMM (including *in situ* melanomas) have been reported with respect to MTX treatment (search conducted on March 18, 2019). Clearly both figures above are ample underestimations of the real-world data. Regrettably, these can therefore not be expeditiously used to answer the research question.

Short and plain language summary

So, in summing up the first section of this thesis, I want to give you a short and plain language summary as to where we stand.

CMM is a potentially fatal cancer type that is increasing in the population. Immunosuppression is associated with an increased risk for CMM as well as a more unfavorable prognosis. MTX has been and is still an anchor drug for the systemic treatment of RA, Pso and PsoA. In RA patients, MTX treatment has been associated with an increased risk for other types of skin cancer. MTX has been linked to LPDs that have been reversed upon withdrawal of the drug. The potential association between MTX and CMM has only rarely been addressed. Nevertheless, an Australian investigation suggested an increased risk for CMM among RA patients treated with MTX. Available pharmacovigilance databases do not contribute to an increased understanding in the subject.

How can we approach an answer to the research question?

Could an association between MTX and CMM have slipped researchers and clinicians' attention over more than 60 years of clinical use? Thinking clearly, if there would have been a clinically relevant association between MTX and CMM, it is safe to say that vigilant physicians would have

discerned clinical patterns over the decades since the drug was first developed. Logically, if there would have been a clinically important association, we would have known already. *Or would we?*

Clearly, the numbers from both databases in the previous paragraphs, represent a significant underreporting and, using these databases can hardly help solve the research question at hand. A prospective cohort investigation could have been performed, but would have consumed tremendous amounts of resources and time. To get closer to an answer, we are therefore left to retrospective real-world data that can be accessed through registers. Importantly, the data obtained through such registers, can never replace the prospectively collected data obtained in prospective clinical trials. The data from registers can be inherently fuzzy, incomplete, and inevitably less well-documented and validated. In this context it is important to talk about the Swedish registers. I will do this in the methods section, but first I would like to give you a brief introduction to epidemiology and epidemiological investigations.

1.11 EPIDEMIOLOGY AND PHARMACOEPIDEMIOLOGY

The word epidemiology derives from the Greek words, *'epi',* which means upon or among and, *'demos',* which refers to the people. Epidemiology is the field of medicine that investigates distribution of a disease or other health factors within a population. An important factor within epidemiology is measuring the incidence of a disease and how incidences change over time. Incidence is defined as the proportion of cases observed during a specific time period. Incidence is usually measured as the number of new cases per 100,000 person-years. As an example, CMM incidence has increased in Sweden over the past decade (i.e. the proportion of the population that get CMM is increasing). Principally, epidemiological research revolves around investigation of possible association between exposures and outcomes. To investigate this, descriptive as well as analytical approaches may be used. Pharmacoepidemiology is the study of the use and effects of drugs and other medical devices in large numbers of people.[2] In the text below, let me shortly present a selection of different types of medical investigations.

Case report

A case report is a medical publication that reports detailed demographic and medical

data on one patient where a possible association between an exposure and a disease has been observed. The case report is usually accompanied by a general discussion and placed into a context of other published cases. Although case reports *per se* are not considered epidemiological investigations, they are important for hypothesis generation and might prove useful for the scientific community. Nevertheless, and perhaps needless to say, observations made in a single case report should not automatically be generalized to the population and therefore it is considered the lowest form of clinical evidence.

Case-series

A case-series is another descriptive investigation that includes more patients that all share the same exposure and where an outcome is observed. A case-series is an empirical observation of cases in which the scientific research question was not thought of *a priori*. Thus, these investigations do not involve hypothesis testing. Nevertheless, case-series can be particularly useful for constructing further investigations. Importantly, as for case reports, no controls without the exposure are included, making it hard to draw generalizable conclusions.

Case-control investigation

This type of observational study is the first that has an analytical approach (i.e. includes hypothesis testing). The researcher starts by identifying all the cases from a population. The cases are patients with an outcome of interest (usually a disease). In the next step, controls are selected. Controls are defined

as individuals without the outcome. Then, exposures are compared between the groups which yields an odds ratio (OR), which is a measure of association between an exposure and an outcome. This type of investigation is particularly useful when the outcome is rare and is very useful in outbreak investigation when it is important to swiftly identify the exposure. Moreover, case-control investigations are very useful in pharmacoepidemiology. Importantly, this kind of investigation is usually more time-saving and less costly as it limits the analysis to a limited number of patients. This type of approach was selected for *Papers V* and *VI*.

Cohort investigation

In statistical terms, a cohort is defined as a group of individuals that share a defined characteristic. Cohort investigations may be prospective (observing the group of patients from a specific date and onward) or retrospective (looking back at historical data). Although reducing the opportunity to obtain important data, when there is only access to registry data in health care registries, the researcher is usually limited to conducting retrospective analyses. Contrary to case-control studies, cohort investigations measure the exposure and then analyze the subsequent risk of outcome. An important advantage of this type of investigation is that the incidence of the cohort can be measured. Moreover, this incidence can be compared to the one in the background population and/or to a corresponding cohort with patients without the exposure of interest. This approach was selected for *Papers I-IV*.

Randomized controlled trial

A randomized controlled trial (RCT) is the gold standard for clinical trials. The background for conducting a RCT is usually introduction of a new treatment. Patients are randomly selected to receive the new treatment, established treatment or placebo. Ideally, the RCT is double-blinded which means that the investigators as well as patients are unaware of the type of treatment given during the study. This type of investigation reduces the risk for bias.

Confounder

A confounder (also referred to as confounding factor or confounding variable) is a variable that influences both the dependent and independent variable. Therefore, a confounder is a factor that explains all or part of the difference between the measure of association and the measure of effect. A confounder can be an exposure, intervention or treatment. When conducting epidemiological research, discussing and identifying potential confounders is essential.

of melanocytes in nests). If a lesion is melanocytic, the overall aim is to decide whether the lesion is benign (nevus) or malignant (CMM). Benign lesions are left for patient self-monitoring, whereas lesions suspected to be malignant need to be excised. If the dermoscopic diagnosis of a lesion falls between the clinical decision of benign or malignant, the lesion is referred to as undeterminable. In this case, dermoscopic follow-up (in selected cases) or excision may be performed.

FIGURE 8a Clinical image of an *in situ* melanoma.

FIGURE 8b Dermoscopic image of the same lesion.

FIGURE 9a Different examples of dermoscopes.
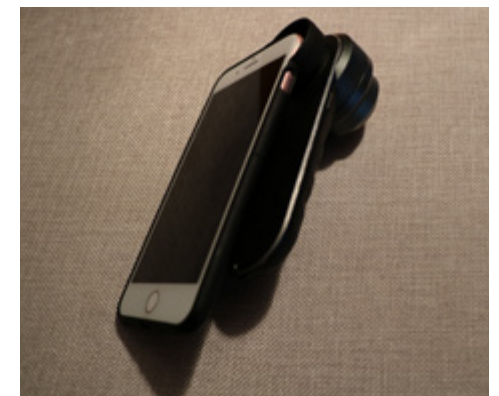
FIGURE 9b A dermoscope attached to a smartphone.

1.12 DERMOSCOPY

To facilitate the understanding of *Paper VI*, let me give you a brief introduction to dermoscopy. Dermoscopy (also often referred to as dermatoscopy) is a valuable tool in everyday clinical Dermatology. As clearly demonstrated from figures 8a and 8b, a dermoscopic image of a pigmented lesion gives a more detailed view compared to the naked eye. A dermoscope is a type of loupe (often with 10x enhancement) equipped with a standardized light source usually with polarized lighting. A dermoscope is particularly useful in the examination of pigmented skin lesions and significantly outperforms the naked eye in examination of suspected

CMMs.[140-142] Conveniently, the device can be attached to a camera setup including smartphones, making it easy to obtain digital images that can be attached to the patient journal for review (Figures 9a and 9b).

To assist physicians in their analysis, specific dermoscopic diagnostic algorithms have been developed to simplify the evaluation of pigmented lesions which, at times, can be challenging. The algorithms vary from clinic to clinic and even between physicians. At our department, the pattern analysis model is often used. In short, the first step is deciding whether a lesion is melanocytic (i.e. has an increased number

When examining a melanocytic lesion, the clinician evaluates if there are melanoma-specific criteria present. A schematic illustration of the melanoma-specific features presented in the pattern analysis algorithm is shown in Figure 10. Moreover, and perhaps needless to say, there is an interobserver variability when assessing these criteria.[143]

Including an article with dermoscopy as the main focus in this thesis was not a part of my original plan. As *Papers I-V* had only revolved around the main research question, my supervisors wanted to challenge me with another project. Then, the idea to specifically address the dermoscopic appearance of CMM in immunosuppressed individuals emerged. One perhaps more obvious path would have been to investigate the dermoscopic features of CMM in patients with ongoing or a history of MTX treatment. Unfortunately, this project proved difficult as no specific code for MTX treatment is included in our journal system. On the other hand, as OTRs are followed at our department, the idea to investigate the dermoscopic features of their CMMs was chosen instead. Although MTX is not specifically used among OTRs, these patients have several other immunosuppressive pharmaceutical agents in order to prevent organ rejection. Therefore, *Paper VI* was of a more exploratory nature.
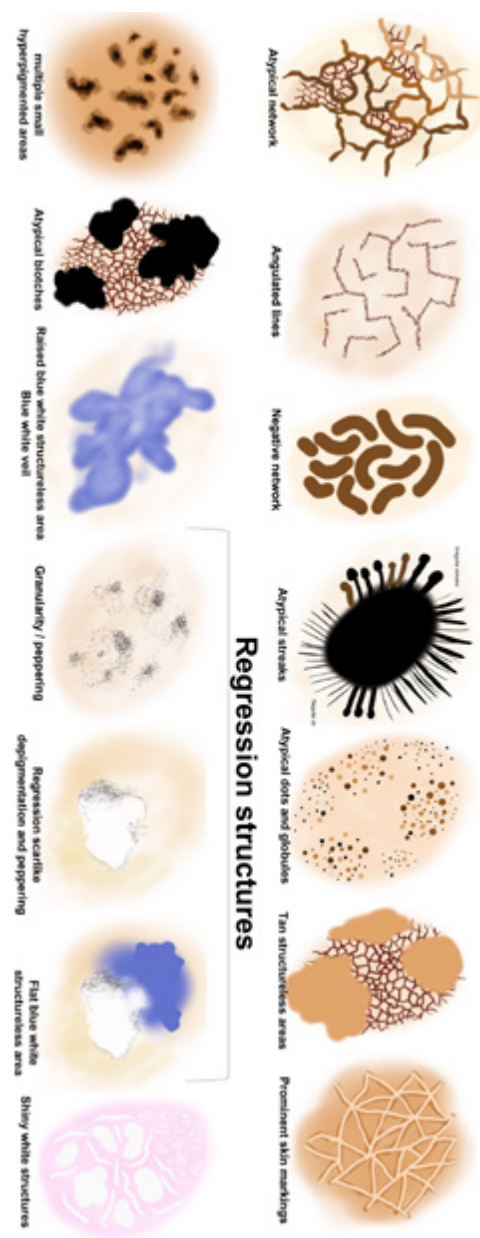
FIGURE 10 Melanoma-specific features from: Revised two-step algorithm. (2018, September 7). *dermoscopedia*. Retrieved, June 13, 2019 from https://dermoscopia.org/w/index. php?title=Revised_two-step_algorithm&oldid=13449

2

2 AIM

The primary research aim of this thesis was to investigate whether there is an association between MTX exposure and risk of CMM in a Swedish population. The secondary aim was to investigate whether CMMs in OTRs have a different set of dermoscopic criteria compared to those of non-OTRs.

THE SPECIFIC AIMS OF THE STUDIES INCLUDED WERE:

• *To investigate if patients without a history of CMM exposed to MTX have an increased risk for CMM compared to unexposed individuals.*

• *To investigate whether a dose-response relationship exists between MTX and the risk for CMM (i.e. do higher accumulated doses increase the risk for CMM).*

• *To investigate if the melanoma characteristics at diagnosis and melanoma mortality differ between MTX-exposed and MTX-unexposed patients.*

• *To investigate if patients with a history of CMM who were exposed to MTX after a first diagnosis of CMM have an enhanced risk for a second primary CMM compared to corresponding MTX-unexposed patients.*

• *To investigate if Pso patients with a CMM (cases) have had a higher exposure to MTX compared to Pso patients without a CMM (controls).*

• *To investigate if CMMs arising in OTRs display a different set of dermoscopic features compared to those of non-OTRs.*

METHOTREXATE AND
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3

METHOTREXATE AND
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3 METHODS

Background to population and health care registers

All residents in the Nordic countries have equal access to health care. This fact has a particularly important implication for epidemiological research, as all citizens have the same opportunity to be included in the health care registers. This, however, is not the case in an international setting where epidemiological investigations usually are conducted on specific insurance registers, precluding patients unable to afford an insurance plan. Moreover, in the Nordic countries, all citizens have a specific personal identification number which facilitates the linking between registers. Needless to say, robust and reliable population and health care registers with an acceptable capture rate are instrumental when conducting epidemiological research. The Swedish registers related to health care are administered by the National Board of Health and Welfare and the population registers are administered by Statistics Sweden. In the paragraphs below, I will briefly present the registers used for *Papers I-V*.

The Prescribed Drug Register

The Prescribed Drug Register[144,145] contains information on all filled prescriptions from Swedish pharmacies from the start of the register in July 2005. The estimated coverage is close to 100%. Importantly, only filled prescriptions are included. Therefore, a patient that never redeemed a doctor's drug prescription will not be included (primary non-compliance). Nonetheless, and needless to say, having filled a prescription does not automatically mean that the patient takes the drug – something that no register can

ever control. Different regions in Sweden have different clinical routines and prescription practices. Thus, information on drugs that normally are administered in an in-patient setting is therefore less reliable. One significant drawback of the register is that the exact diagnosis that prompted the prescription (for selected drugs) is not included. The usefulness of the register would increase if physicians had to select the diagnosis (or diagnoses) that prompted the prescription. Nevertheless, the clinic from where the drug was prescribed is included which can sometimes act as an indicator of the indication that prompted prescription. The Swedish Prescribed Drug Register was used for *Papers I-V*.

The Cancer Register

The Cancer Register[146] was established in 1958. In Sweden, reporting of incident cancers is mandatory, resulting in an estimated coverage greater than 95%.[147] The register includes, among other variables, information on date of diagnosis and the type of cancer according to the ICD-10 classification (International Classification of Diseases, 10th revision). Both *in situ* as well as invasive tumors are included. The Cancer Register was used for *Papers I-V*.

The Cause of Death Register

The Cause of Death Register[148] provides information on dates and causes of death for all deceased residents from 1961 onwards. The Swedish Cause of Death Register was used for *Papers I-V*.

The Outpatient Register

The Outpatient Register[149] was initiated in 2001 as a new component of the Swedish Patient Register (which also covers virtually all hospital discharges since 1987). The Outpatient Register includes information on diagnoses in non-primary outpatient care, coded according to ICD-10. For each diagnosis, the specific specialty that gave the diagnosis is included. The register is available from the National Board of Health and Welfare. The Outpatient Register was used for *Paper V*.

The Inpatient Register

The Inpatient Register[150] was started in 1964 and covers inpatient hospitalizations (in Sweden) for all Swedish citizens. The date of admission and the date of discharge as well as the main diagnoses and all other diagnoses are recorded. For each hospitalization, the specific specialty that treated the patient is included. The register was used for *Paper V*.

The Population Register

The Population Register[151] is available from Statistics Sweden. The register includes data on residency and dates of immigration and emigration for all people residing in Sweden from 1961 onwards, and coverage is virtually complete. Moreover, data on educational level, income level, civil status, geographical region and country of birth are included if those data are available. The Swedish Population Register was used for *Paper V*.

Important considerations in selecting methodology for Papers I-V

The null hypothesis for *Papers I-V* was that MTX does not influence the risk of CMM. Therefore, in order to challenge the null hypothesis (i.e. find an answer to the research questions) I needed to conduct epidemiologic observational investigations with an analytic approach. Bearing in mind that only retrospective investigations are available, two possible options were considered: *case-control studies* and *cohort investigation* (Figure 11).
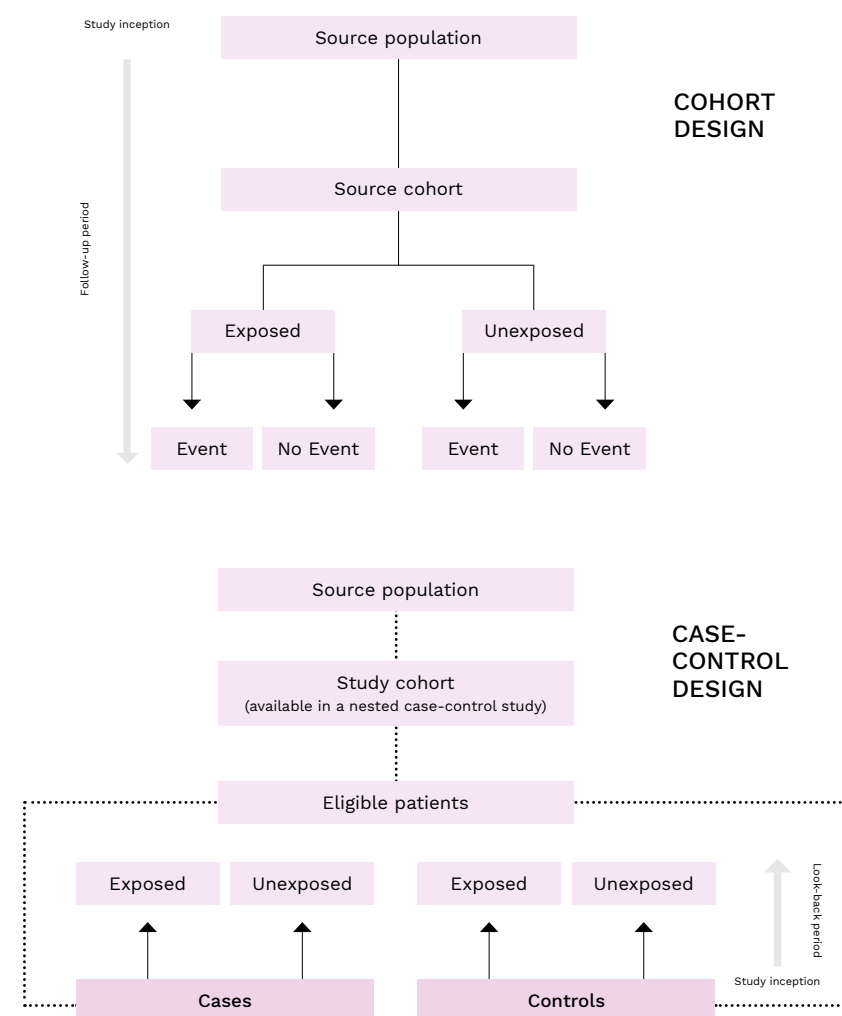

FIGURE 11 A schematic illustration of a cohort investigation and a (nested) case-control investigation.

As mentioned before, a case-control study is an investigation that compares cases with a disease to controls without a disease. The control selection in case-control investigations is an important and delicate step that deserve particular attention. In case-control investigations matching is sometimes used. Matching refers to selection of controls that are similar to the cases. Matching variables used are often age and sex. A common misconception is that matching increases the validity of a study. The role of matching is to increase the efficiency of a study. However, if the controls are matched to the cases, the matching criteria must be true confounding variables. If not, bias can be introduced into the study.[152,153] After the selection of controls, precedent exposure is analyzed in both groups. A case-control setup is often used in pharmacoepidemiology and is often the first approach to consider in order to answer the research question as it is easier logistically, faster and less expensive. However, a limitation is that the incidence rate cannot be calculated. The advantage of including incidence is that it can be compared to the incidence of the background population. Thus, a case-control investigation, is not easily integrated in meta-analysis investigations. One potential strength of conducting a case-control study is that multiple exposures can be investigated simultaneously.

That is why we used a case-control design for *Paper V*. The term "nested" implies that the study cohort is drawn from an enumerated cohort of patients.

Cohort investigations are also frequently used in epidemiological investigations.

In the first step, a cohort of patients that share the same exposure is selected. Then the observed cases in the cohort are calculated. When applying incidences available from the background population, the number of expected cases in the cohort can be calculated. A SIR is obtained when the number of observed cases is divided by the number of expected cases. This approach was conducted by Buchbinder *et al.*[12] Using the incidence rates from the background population you indirectly assume that the vast majority of patients in that population will not have the exposure of interest.

Another option is to include a cohort of individuals that don't have the exposure of interest (unexposed). Ideally, this cohort is as similar as possible except for the exposure of interest. Practically, the unexposed individuals are usually selected with respect to age and sex to the corresponding individuals in the exposed cohort. The exposed and unexposed can then can then be compared to each other with respect to the outcome of interest. One significant advantage of including an unexposed cohort is that the risk for secondary malignancies can be easier to calculate.

Overall, cohort investigations are useful in post-marketing investigations when introducing a new drug and the examination of possible side effects making it an appealing method in this context. Generally, associations demonstrated by cohort investigations are more likely to be causal compared to those observed in case-control investigations. For those reasons, we selected cohort investigations for *Papers I-IV*.

When conducting *cohort* as well as *case-control* investigations, several medical journals require a specific reporting according to a defined checklist (STROBE-checklist). Thus, when preparing an analytical epidemiological investigation all criteria have to be well-thought-out *a priori*.[154]

3.1 PAPER I
Subjects
The study population consisted of all Swedish patients over the age of 18 years who had filled any MTX prescription (*ever-exposure*) from Swedish pharmacies (*MTX-exposed*). For each MTX-exposed patient, five age- and sex-matched individuals that had not been exposed to MTX were included (*MTX-unexposed*).

Design
This was a nationwide, retrospective, registry-based and comparative cohort investigation. The Prescribed Drug Register was used to include all patients with any filled MTX prescriptions in the time period above. MTX-unexposed had filled a prescription of a randomly selected drug excluding MTX.

The MTX-unexposed individuals were randomly selected among those who had received another drug in the same time period ±1 month to the corresponding MTX-exposed individual. The inclusion of unexposed individuals in the same time period allowed roughly the same follow-up time in both cohorts.

The lists were matched to the Cancer Registry and all cases of CMM including *in situ* melanomas were obtained since the register was initiated in 1958. All individuals with a prevalent diagnosis of CMM before MTX-exposure or the corresponding date among the controls were excluded from this analysis. Follow-up was censored at whatever came first: reported date of CMM, end of study follow-up or death. Death dates were obtained from the Cause of Death Register.

Statistical analysis
A Cox proportional hazards regression model was used testing for a difference in hazard for CMM between the MTX-exposed and the MTX-unexposed. The model contained the sex and age group at treatment start as covariates. The proportion of CMM among the MTX-exposed and MTX-unexposed patients was compared, stratifying with respect to sex and age group using the Mantel-Haenszel test. A Poisson test was used to compare the incidence of CMM between the groups during the time period 2005–2014. The incidence rates of CMM in both groups were compared with those of the general population of Sweden

using sex- and age-standardization. Fisher's exact test was used to compare proportions. All tests were two-sided and P < 0.05 was considered statistically significant.

3.2 PAPER II
Subjects
The study population consisted of the same MTX-exposed and MTX-unexposed individuals as in *Paper I*.

Design
The Prescribed Drug Register was used to calculate the accumulated number of filled MTX prescriptions. Information on all filled prescriptions of MTX was available, including route of administration (oral/parenteral) and dose. For the respective MTX-exposed patients, all filled MTX prescriptions were calculated, adding up to a total accumulated dose (in g) during the studied time period. Patients with a missing accumulated dose were excluded from the analysis. The MTX-unexposed individuals were included in some subanalyses as well as mortality analyses.

Statistical analysis
For the primary analysis, only MTX-exposed patients were included. A Cox proportional hazards regression model was used with the time from the first observed filled MTX prescription to the first CMM as the dependent variable. The independent variables used were: sex, age group at treatment start, total accumulated MTX dose and time from first to last filled prescription of MTX during the period 2005-2014.

This last variable was divided into six time periods. The age groups at treatment start were divided into predefined age intervals. The same analysis was repeated within each subgroup of the above six periods between the first and last filled prescription of MTX. The HRs and CIs corresponding to a total MTX exposure of 1 g were calculated for each model.

The overall incidence rates of CMM during the period 2005-2014 and the corresponding SIRs (MTX observed/MTX expected) were calculated and Poisson tests were performed. The expected incidences were computed, keeping the sex and age distribution from the MTX-exposed fixed, but assuming the same underlying incidence of CMM as in the Swedish general population. The above analysis was performed within subgroups divided according to total accumulated MTX dose into different dose groups. Cox proportional hazards regression models were used to compare the time to CMM between the MTX-exposed patients who received their first filled prescription of MTX in 2005 and their corresponding MTX-unexposed counterparts with sex and age group as independent variables. The analysis was separated into five models corresponding to the abovementioned dose intervals. In each model, the MTX-exposed individuals were compared with their respective MTX-unexposed counterparts. A Cox proportional hazards model was used where the MTX-exposed patients with exclusively parenteral MTX administration were compared with their corresponding

MTX-unexposed subjects with respect to CMM risk, with sex and age group as independent variables. All tests were two-sided and P < 0.05 was considered statistically significant.

3.3 PAPER III
Subjects
The study population consisted of the same MTX-exposed and MTX-unexposed individuals as in *Papers I* and *II*.

Design
For this investigation, the melanoma-specific mortality was compared between MTX-exposed and MTX-unexposed individuals that developed a CMM. Information on the cause of death was obtained from the Cause of Death Register. The stage of the CMM (TNM) was obtained by the Cancer Registry.

Statistical analysis
Mantel-Haenszel's test was used to compare melanoma mortality adjusted for melanoma stage at diagnosis. Fisher's exact test was used to compare proportions. All tests were two-sided and P < 0.05 was considered statistically significant.

3.4 PAPER IV
Subjects
The study population consisted of all Swedish patients over the age of 18 years that had been filled prescriptions of MTX from Swedish pharmacies (*MTX-exposed*) and had any prevalent CMM prior to MTX exposure. A corresponding group of MTX-unexposed

individuals was obtained.

Design
Paper IV was designed as a retrospective, registry-based and comparative cohort investigation. It was conducted to evaluate whether MTX treatment after a diagnosis of CMM increased the risk for a consecutive primary CMM.

Statistical analysis
A Cox proportional hazards regression, controlling for age group, sex, and time interval from the first CMM was performed. The Kaplan-Meier estimates for the 5-year risk of a consecutive primary CMM was calculated in both groups. Fisher's exact test was used to compare proportions. All tests were two-sided and P < 0.05 was considered statistically significant.

3.5 PAPER V
Subjects
The subjects included in *Paper V* were drawn from an enumerated Swedish cohort of Pso patients. In order to be included in this cohort, a patient had to be diagnosed with Pso at least twice and at least once by a dermatologist between January 2001 and December 2016. For *Paper V*, specifically, only patients with a first Pso diagnosis prior to July 2005 were included.

Design
This was a nested case-control investigation from the cited cohort above. Patients with CMM diagnosed between January 1, 2006 to December 31, 2016 were designated as *cases*.

Prior to CMM, cases had to be cancer-free. For each case, 10 controls were matched on age, sex, income and educational level. The controls were selected on the corresponding date of melanoma diagnosis to allow for the same observation period.

Statistical analysis

Conditional logistic regression models were used and crude as well as adjusted ORs were calculated.

3.6 PAPER VI

Subjects

All OTRs with a CMM and an available dermoscopic image were selected (*cases*). To each case, age- and sex-matched non-OTRs also diagnosed with a CMM and with an available dermoscopic image were randomly selected (*controls*). All patients were followed up at the Department of Dermatology at Sahlgrenska University Hospital.

Design

Study VI was a single-centre, blinded, retrospective and comparative case-control study. CMMs in the OTR group and in non-transplanted immunocompetent patients were selected from patient medical records. For the OTR group, all available cases were identified in the time period 2007 to 2018. To each case, four to five age- and sex-matched individuals were randomly selected.

All available images from above were presented to two blinded dermatologists. Dermoscopic criteria were evaluated according to the most recent version of the pattern analysis algorithm.

Statistical analysis

Fisher's exact test was used for two-sample tests. Cohen's kappa (κ) was used for interobserver agreement. All tests were two-sided and $P < 0.05$ was considered as statistically significant.

3.7 ETHICAL CONSIDERATIONS

Papers *I-VI* had ethical approvals from the Regional Ethical Review Board of Gothenburg prior to initiation (approval numbers: 461-*15*, 911-*17* and 283-*18*).

As *Paper VI* included individuals from a highly selected patient group (ie. OTRs with a CMM diagnosis), a discussion within the research group was conducted not to indirectly reveal the identity of these patients. As only eight patients with nine CMMs were included, the blinding process was crucial. In the manuscript, only dermoscopic images are available as to guarantee patient anonymity.

4

METHOTREXATE AND RISK OF CUTANEOUS MELANOMA

Sam Polesie

4 RESULTS

4.1 PAPER I

In the time period of August 1, 2005 to December 31, 2014, approximately 100,000 individuals filled prescriptions of MTX from Swedish pharmacies. The MTX-unexposed group consisted of approximately 500,000 patients who filled a prescription of a randomly selected drug other than MTX. All patients with any CMM prior to the start of the observation period were excluded from the analysis. In the MTX-exposed group, 591 patients (0.58%; 95% CI 0.54–0.63) developed CMM in the time period above. In the MTX-unexposed group, the corresponding number was 2506 (0.50%; 95% CI 0.48–0.52), which was significantly fewer than in the MTX-exposed group (P < 0.001). The distribution of invasive and *in situ* melanomas did not differ between the groups. The risk increase in the MTX-exposed group was sustained (P < 0.001) when both the MTX-exposed group

and the MTX-unexposed group included only the patients who were prescribed medication in 2005 and therefore had the longest follow-up period. When the analysis was separated into sex and age group at treatment start, a significantly increased risk for CMM in the MTX-exposed group was observed only in women older than 70 years. To address possible confounding by indication, a subgroup analysis was performed. The subgroup comprised 31% of all the patients in the MTX-exposed group and included patients that had exclusively been prescribed MTX by a dermatologist and a rheumatologist. When comparing the two subgroups with their corresponding MTX-unexposed patients, no significant differences in risk for CMM were seen.

The observed incidences for CMM in the respective groups were compared with

the expected numbers using national melanoma statistics from 2005 to 2014. In the MTX-exposed group the incidence for CMM (including *in situ* melanomas) in the time period 2005–2014 was 99.5 per 100,000 person-years. The corresponding number for the MTX-unexposed group was 93.2. There was no significant difference between the incidences over the entire period 2005–2014 between the patients in the MTX-exposed and patients in the MTX-unexposed groups (P=0.07). Moreover, the observed incidence rates in the MTX-exposed and MTX-unexposed groups were compared with the expected numbers using year-, age- and sex-specific incidences in Sweden. SIRs comparing MTX-exposed patients with their corresponding MTX-unexposed patients did not differ significantly from unity.

4.2 PAPER II

The risk of CMM did not significantly depend on MTX dose (P=0.41). The model yielded a HR of 1.02 (95% CI 0.97–1.08) for 1 g of total MTX exposure. No significant association with respect to dose was found for the risk in any subgroup when the patients were divided into groups with respect to time from the first to the last MTX exposure. When the analysis was repeated for subgroups of patients with an exclusive prescription from a rheumatologist or a dermatologist, respectively, no significant dependence between the risk of CMM and the accumulated dose was observed in either subgroup.

The observed and expected incidence rates of CMM within different intervals of the total accumulated MTX dose were compared. A significant risk increase was seen for MTX-exposed individuals compared with the Swedish population for the dose intervals 2-4 g; 4-6 g and 6-8 g. However, no risk increase was observed for the groups ≤ 2 g and > 8 g. In a subanalysis, patients who had a first prescription of MTX in 2005 were compared with their MTX-unexposed counterparts with respect to risk of CMM. A significant difference in the risk of CMM between the MTX-exposed and unexposed individuals was observed in the subanalyses in which MTX-exposed patients had a total accumulated dose of 4-6 g and 6-8 g. However, no significant differences between MTX-exposed and unexposed individuals were observed in the subanalyses corresponding to ≤ 2 g; 2-4 g and > 8 g.

Patients with an exclusively parenteral MTX exposure (n=3774) were compared with their respective MTX-unexposed patients (n=18,699) for a difference in risk of CMM. No significant difference was found between parenteral MTX-exposed and unexposed patients. Finally, comparing the overall mortality after the first filled prescription, including all causes of death between the MTX-exposed and MTX-unexposed, yielded an increased mortality for the MTX-unexposed among men > 40 years and an increased mortality for MTX-exposed among women aged ≤ 50 and > 70 years.

4.3 PAPER III

Among the MTX-exposed patients with a first CMM in the time period 2005–2014

(n=591), 38 had CMM as the reported cause of death, 6.4% (95% CI 4.6–8.7%). The corresponding proportion among the MTX-unexposed patients (n=2506) was 3.4% (95% CI 2.7–4.1%; P=0.0013). No significant differences were found between the groups in the distribution of sex, age and pathological stage at CMM diagnosis. However, when comparing melanoma-specific mortality, adjusting for pathological stage at diagnosis, a higher mortality among the MTX-exposed was detected, OR 1.9 (95% CI 1.2–3.0; P=0.003). When analyzing the entire cohort, the cancer-specific mortality (including all cancers) was significantly lower among MTX-exposed (3.6%) compared to MTX-unexposed (4.0%) (P < 0.0001). In two subanalyses, the above comparison was repeated for individuals with an exclusive MTX prescription from a dermatologist (n=10,399) or a rheumatologist (n=39,701) and the respective MTX-unexposed individuals. For the individuals with a CMM diagnosis in the Dermatology group, there was no significant difference in melanoma-specific mortality between the MTX-exposed and MTX-unexposed individuals. However, for CMM diagnosed in the Rheumatology group, a higher mortality among the MTX-exposed was detected when adjusting for pathological stage at diagnosis, OR 2.2 (95% CI 1.0–4.8; P=0.039).

4.4 PAPER IV

Among the MTX-exposed and MTX-unexposed, 1216 and 6696 patients, respectively, had a history of CMM and were included in the analysis. In the MTX-exposed

group, 105 of 1216 patients (8.6%; 95% CI 7.1-10.4%) developed a consecutive CMM. In the MTX-unexposed group, the corresponding number was 553 of 6695 patients (8.3%; 95% CI 7.6-8.9%; P=0.65). No significantly increased risk for a consecutive CMM in the MTX-exposed group compared to the MTX-unexposed group was observed (HR 1.0; 95% CI 0.8-1.2; P=0.98). The Kaplan-Meier estimates for the 5-year risk of a consecutive CMM was 5.7% (95% CI 4.3-7.1%) in the MTX-exposed group and 6.1% (95% CI 5.5-6.7%) in the MTX-unexposed group.

4.5 PAPER V

Of 220 Pso patients with CMM, 51 (23%) had filled prescriptions for MTX. Among 2200 controls, 493 (22%) had filled a prescription of MTX (crude OR 1.0; 95% CI, 0.7-1.5). In multiple conditional logistic regression analysis, no association between MTX exposure (ever use) and risk for CMM were observed (adjusted OR 1.0; 95% CI, 0.7-1.4). Moreover, no indication of a dose-response association was observed.

4.6 PAPER VI

In the OTR group, three invasive melanomas and six *in situ* melanomas were identified in eight male patients (age range at melanoma diagnosis: 47 to 74 yrs). The control group included 24 invasive melanomas and 16 *in situ* melanomas in 34 male patients (age range at melanoma diagnosis: 46 to 75 yrs). In the OTR group, 33% of melanomas were invasive and, in the control group, the corresponding number was 60% (P=0.27).

Among all cases, 43% were on the trunk, 15% on the head and neck, 15% on the upper extremities and 8% on the lower extremities. There was no significant difference in the distribution of localization between the OTR group and the controls.

After adjustment for multiple comparisons, no melanoma-specific structures were more prevalent in the OTR group than in the control group for any of the features ($P > 0.05$ for all features and for both observers). This also applied to regression structures and atypical vascular patterns. Moreover, facial melanomas in both groups displayed the same features.

Among melanoma-specific structures there was a moderate interobserver agreement; κ=0.54 (95% CI 0.44–0.63). Regression structures and atypical vascular patterns had fair to moderate interobserver agreement; κ=0.44 (95% CI 0.24–0.64) and κ=0.45 (95% CI 0.28–0.62), respectively.

5

5 DISCUSSION & METHODOLOGICAL CONSIDERATIONS

5.1 PAPER I

Methodological considerations

One significant methodological consideration is the method for selecting the MTX-unexposed individuals in *Papers I-IV*. As these papers used the same cohort of patients, the discussion below applies to all of these investigations.

Selection of the methotrexate-unexposed individuals

The MTX-unexposed individuals were age- and sex-matched and had filled a prescription of one randomly selected pharmaceutical drug within one month compared to the corresponding MTX-exposed individuals. One advantage of using this selection strategy, is that you know for certain that the MTX-unexposed individuals had had contact with the health care system (i.e filling a prescription is usually a consequence

of an appointment with a health care professional). When seeking health care, you probably have trust in the health care system and you would probably be willing to use their services again. Nevertheless, the MTX-unexposed individuals were not selected from a population register, which certainly would have been a more appropriate option. Moreover, only one single dispensation of one drug was obtained among the MTX-unexposed, thus only minimal information is known about this cohort. The MTX-unexposed individuals were selected on use of pharmaceutical drugs other than MTX. Thus, we know that MTX-unexposed individuals were not exposed to MTX for the whole observation period (i.e. any MTX exposure would have meant inclusion in the cohort of MTX-exposed). Although this would only have a minimal impact, this selection would in fact limit to pooling of

eligible MTX-unexposed individuals. In retrospect, it would certainly have been wiser to have drawn the MTX-unexposed individuals from the general population using available population registers. Moreover, since CMM incidence varies between different counties within Sweden, geographic matching would have been valuable.

Other methodological considerations

Data on several demographic details including educational level and country of birth were not retrieved, which certainly was an epidemiological drawback and a methodological limitation. Moreover, data on emigration were not retrieved.

Another important limitation is that only data on MTX were obtained, leaving out other concomitant drugs. Moreover, data on co-morbidities were not retrieved. While attempts were made to adjust for confounding by indication, the exact diagnosis that prompted prescription was unknown.

For this investigation, data on several known melanoma risk factors were missing including: family history of melanoma, sun exposure history (including sunburn in particular at a young age), sunscreen use, skin phototype and nevus count. Moreover, exposure to pharmaceutical drugs before July 2005 were not available.

Another important factor that needs particular attention is confounding by indication. In other words, having filled a MTX prescription means that a patient has a specific disease that can be treated with MTX. This disease, may, intrinsically, increase the risk for CMM. Significantly, there are no indications that RA nor Pso increase the risk for CMM in Swedish investigations.[155-157] Nevertheless, the most suitable MTX-unexposed individuals should ideally have had the same disease as the MTX-exposed. This would have reduced the confounding by indication.

Another, confounding variable is the surveillance bias brought on by having a chronic autoimmune or inflammatory disease. Patients in the MTX-exposed group clearly have a disease that needs systemic drugs. Therefore, it is expected that patients in this group have more contact with the health care system compared to the MTX-unexposed individuals. Therefore, it cannot be excluded that some of the CMMs found among the MTX-exposed were identified due to increased surveillance.

General discussion

The primary outcome of interest for *Paper I* was a comparison between the MTX-exposed and MTX-unexposed individuals. However, comparison of the incidences between the respective groups were performed and did not demonstrate any significant risk increase. Nevertheless, although not significant, the results still pointed in the same direction as the main analysis. Moreover, the respective groups were compared to the incidences of the Swedish population. When analyzing these results separately, there was no indication as to MTX enhancing the risk for CMM.

When reviewing the main analysis, it is necessary to discuss how the results from it should be interpreted. The primary analysis yielded a HR between MTX-exposed and MTX-unexposed of 1.17 (95% CI 1.08-1.26, P=0.0006). The number **1.17** relates to a measure of the association observed. Clearly, the main analysis generated a significant result, but the important question is of course how should **1.17** be interpreted in a clinical setting?

This key question relates to one of the Bradford Hill epidemiological viewpoints presented in the beginning of this thesis (section 1.4), namely quantitative strength of an association. Conventionally, associations with a relative risk < 2.0 are generally considered to be weak.[2] Others have suggested that a relative risk > 3 to be convincing for more severe adverse events[158], which certainly indeed would apply to CMM.

The HR of 1.17 with the accompanied CI should also be compared to other investigations performed (criteria for consistency). This brings us back to the Buchbinder investigation.[12] As these authors compared the observed with the expected numbers of CMMs (using the background population), they presented a SIR of 3.0 (95% CI 1.2-6.2). Due to the different analyses conducted, '1.17' and '3.0' are not easily compared. Nevertheless, both of these point at the same direction. Having said that, these numbers only indicate statistical correlations and should of course be interpreted in the light of all the positive effects that MTX has to

these patient groups. The number needed to harm estimated in our investigation was 1250, which, in plain language, means that 1250 individuals needed to be exposed to MTX in the time period in order to explain one single case of CMM. In contrast to this, after 16 weeks of treatment with MTX, approximately 40% of patients with Pso experience a 75% reduction of a psoriasis severity specific score.[159] Futhermore, MTX is still the cornerstone treatment of RA with good clinical response when tolerated.[160]

5.2 PAPER II

Methodological considerations

Assessing a dose-response association proved to be challenging. Rather than measuring the accumulated MTX doses, the numbers of filled prescriptions could have been obtained instead. This would perhaps have been clinically more relevant and more comprehensible for the reader. Patients with only a single dispensation of MTX (i.e. a trivial exposure) surely only had a single dispensation perhaps due to side effects or lack of efficacy. Due to the study design, it is impossible to know to what extent these patients went on to try other alternative therapies. Noteworthily, no induction period was required for this analysis. An induction period is a time that must pass before onset of exposure and the outcome. As an example, if a patient would develop a CMM only one week after MTX onset, it would be inappropriate to blame MTX for causing that CMM. In similar studies where an association between an exposure and a cancer is investigated, an induction period of at

least one year is often used. Introduction of an induction period can also be introduced in sensitivity analysis to test for robustness of an association. Induction periods are important for the temporality (i.e. the exposure needs to precede the outcome). The reason why an induction period was not used was due to the fact that only prescription data prior to July 2005 was unavailable. Therefore, most of the patients with filled MTX prescriptions in 2005 obtained in our investigation most likely had an underestimated total number of MTX prescriptions. Nevertheless, despite this, the introduction of an induction period should in hindsight, have been investigated.

General discussion

This is the first publication that specifically has investigated a possible dose-response investigation between the risk of MTX and CMM. The data was somewhat conflicting as different analyses pointed in different directions. Overall, no conclusive indication for a dose-response association was observed. This certainly brings doubt to whether the association observed in *Paper I* was causal.

5.3 PAPER III
Methodological considerations

When interpreting the results from *Paper III* it is important to remember that even though an enhanced risk for melanoma-mortality was observed among MTX-exposed, this do not necessarily mean that MTX intrinsically was responsible. The same arguments for confounding by

indication mentioned previously can be used. Patients with a chronic autoimmune or inflammatory disease might have a worse prognosis for CMM and potentially this does not necessarily have anything to do with drug exposures at all.

General discussion

Because no clear-cut dose-response relationship was observed in *Paper II*, it is important to interpret the results in *Paper III* with caution. Nevertheless, it is interesting to see that when adjusting for melanoma stage at diagnosis, an enhanced risk for melanoma-mortality was observed among the MTX-Rheumatology group but not the MTX-Dermatology group.

5.4 PAPER IV
Methodological considerations

While *Paper IV* used the same cohort as for *Paper I-III*, the results are potentially more reassuring. Although a lot of important data are lacking, as already discussed above, all patients included in *Paper IV* share a common feature – they have all had a CMM. Having a history of CMM is one of the most significant risk factors for having a second CMM. This means that most of them are more vigilant and most likely would react should any suspicious mole change or new lesion appear.

General discussion

While *Paper I-III* are interesting, *Paper IV* can prove particularly useful in a clinical setting when a patient develops a CMM while on MTX therapy or when MTX onset is considered for such a patient with a history of

CMM. We found no indications that MTX after CMM enhanced the risk for a new primary CMM. At the end of the day, even though this particular patient group is relatively rare, it is reassuring to have investigated this. Moreover, the results would certainly have been useful for the patient that, in fact, was the inspiration to this thesis. Interestingly, the article has been included in Danish guidelines made by the Danish Dermatology Society regarding CMM.[161]

5.5 PAPER V
Methodological considerations

Paper V was performed to overcome several of the difficulties and methodological flaws that were mentioned above. In fact, we were advised to conduct a case-control investigation addressing this issue at my half-time seminar. This paper was conducted as a nested case-control investigation from a Swedish cohort of patients with Pso. The term nested refers to the fact that all patients (cases and controls) were selected from this enumerated cohort. Only previously cancer-free patients were included. The only difference was that cases had a CMM whereas controls were cancer-free on the date on which the cases were diagnosed with CMM. Several demographic data including comorbidities were obtained from available registers. The investigation was performed among patients with a diagnosis of Pso just to control for confounding by indication discussed previously.

General discussion

Since the inclusion criteria were rather

narrow, only 220 cases were selected. This is a rather small number of patients and only 51 of these had filled MTX prescriptions. Despite this, the results are reassuring and, in fact, confirm the results obtained in *Paper I* in which no enhanced risk for CMM was observed for those patients who only filled MTX prescriptions from a dermatologist.

5.6 PAPER VI
Methodological considerations

Only two observers were included when evaluating the lesions. Including more observers would perhaps have been more suitable. Moreover, consensus evaluation where all physicians could discuss and choose the specific melanoma criteria would have been interesting.

General discussion

While the results presented in *Paper VI* are reassuring, the investigation was only a small pilot study. Therefore, it is important not to generalize the results given a similar clinical real-life situation, but rather see the results as an invitation to further research. Clearly, a multicenter prospective trial including various patients with and without different types of immunosuppression including OTRs would have been a logic sequel to this investigation.

A central aspect about this investigation that needs particular mention is the fact that all analyzed dermoscopic images were shared with the scientific community. In our opinion, this is a key feature when conducting this kind of research. Unfortunately, it is still

a rare event. Nowadays, the online distribution of medical journals is almost exclusively used and several publishers have discontinued traditional paper formats. Therefore, including an e-supplement where all analyzed images are included should in my opinion be compulsory for peer-review approval of the publication as long as the patients can't be identified and remain completely anonymous. In this setting, a large pool of "validated" images can quickly be obtained by the research community and possibly included in databases and included in open source databases used to develop artificial intelligence algorithms.

6

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6 CONCLUSION

PAPER I

For patients without a history of CMM, MTX has, at most, a clinically insignificant effect on increasing the risk for CMM.

PAPER II

No conclusive nor convincing signals were observed for a dose-response association between MTX exposure and the risk for CMM.

PAPER III

Patients with MTX exposure and CMM had a worse melanoma-specific survival, which warrants further attention.

PAPER IV

There are no indications that MTX exposure after a CMM increases the risk for a subsequent primary CMM.

PAPER V

MTX treatment for Pso patients does not increase the risk for CMM.

PAPER VI

In this pilot investigation, there were no indications that CMMs arising in OTRs had different dermoscopic features.

7

7 WHAT HAVE I LEARNED?

Throughout this project, I have learned much and it is not easy to summarize all the new insights in a single paragraph. But, in the text below, I will try to mention some more personal aspects.

Profoundly, I have learned the complexity and inherent difficulties working with population-based registers. Having said that, I have realized what true potential as well as limitations our health care registers display. I have learned that research is a maturing process and that scientific questions really open up to new questions that you did not think of beforehand.

I have had several opportunities to communicate and clarify my research to other peers both orally as well as in manuscript format and I feel that I have improved in this skill over time. From day one, I have regarded my Ph.D. project as an opportunity to learn. To others I have compared it to the process of taking a driver's license. It has been rewarding to have come up with my own theme for a dissertation with relevant roots in clinical practice. Firstly, I did not to realize that, overall, it was quite rare that a Ph.D. student and not the supervisor has come up with the direction of the Ph.D. project. However, there have been times where I have felt too engaged in the scientific question, perhaps, since it was a personal project. As an example, when other researchers gave critique, I was initially more easily offended than I should have been. Now, however, I believe I have learned to see critique as a fruitful way to improve further scientific questions. It has been stimulating to share the results to the scientific community. I have understood how the peer-review system works and how all peers collaborate to help improve manuscripts and ultimately challenge the *status quo* through medical publications.

I believe this has provided me with an in-depth understanding on how to communicate critique to scientific questions. So, when I recently was invited to review a manuscript myself, I really saw it as a good opportunity to give critique in a constructive and encouraging way.

Overall, I have learned to be humble to medical research.

8

8 FUTURE PERSPECTIVES

As I have singled out epidemiological research as one of my main interests, it is my wish to linger in this field. As MTX has been linked to an increased risk for NMSC in other populations, this would be an important and logic future investigation. After that investigation, I think I have to take a rest from MTX and it would be stimulating to put other commonly used drugs used in a dermatological context under scrutiny. Moreover, I would certainly be open-minded to Nordic collaborations within pharmaco-epidemiological investigations important for everyday clinical practice.

I guess this is a section where I can share some of my personal ideas and thoughts. So here are some of them.

Thinking some years ahead, I sincerely hope we will make better use of our national registries and allow automated interactions between them. Automatic linking between registries would generate a bank of big data and the use of artificial intelligence could aid in finding unintuitive and unexpected associations that warrant further investigation by clinicians. Specifically, when a patient receives a CMM diagnosis, I have never heard any of my colleagues giving priority to writing a side effect report for all the drugs that the patient takes. It simply takes too much time and effort. However, I think everybody would agree that this information is pivotal. Therefore, it would be a tremendous help if all pharmaceutical drugs were exported automatically to a side effect database.

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All investigations that directly involve the patient such as clinical trials and interventions must have ethical approval. However, when appropriately anonymized, registry-based data should not be hard to get hold of as an easier access will ultimately help improve how we practice medicine. Rather than writing ethical approvals for retrospective epidemiological research, it would be more desirable to have a similar thing as a driver's license to conduct these kind of retrospective investigations. Needless to say, data should never be presented so that it can be linked to one single patient.

The step from idea to study protocol is usually not a timely one. However, when researchers request data from authorities you need to be patient. When we ordered data the second time it took over a year after the request was sent to the final delivery of data. This hurdle is so unnecessary and seriously affects the conduction of this kind of investigation. Allowing the responsible researcher(s) to gain reading access to relevant registers through secure and validated servers at dedicated computers would enhance the productivity enormously.

Obviously, this suggestion would require politicians and policymakers to alter the law system, but I believe it would be a motivated investment for our society and more significantly pave the path for upcoming generations.

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